

NOVEL ANTIANGIOGENIC PEPTIDES, POLYPEPTIDES ENCODING SAME  
AND METHODS FOR INHIBITING ANGIOGENESIS

This application is a continuation-in-part of U.S. Serial No. 08/851,350 filed May 5, 1997, now pending, which is a continuation in part of U.S. Serial No. 08/832,087 filed April 3, 1997, now pending, which is a continuation-in-part of Serial No. 08/643,219 filed May 3, 1996, now pending.

Technical Field

The present invention relates to the field of peptide chemistry. More particularly, the invention relates to the preparation and use of peptides containing amino acid sequences substantially similar to the corresponding sequences of the kringle 5 region of mammalian plasminogen, pharmaceutical compositions containing the peptides, antibodies specific for the angiostatin receptor, means for angiostatin detection and measurement, cytotoxic agents linked to angiostatin proteins and treatment of diseases which arise from or are exacerbated by angiogenesis.

Background of The Invention

Angiogenesis, the process by which new blood vessels are formed, is essential for normal body activities including reproduction, development and wound repair. Although the process is not completely understood, it is believed to involve a complex interplay of molecules which regulate the growth of endothelial cells (the primary cells of capillary blood vessels). Under normal conditions, these molecules appear to maintain the microvasculature in a quiescent state (i.e. one of no capillary growth) for prolonged periods which may last for as long as weeks or, in some cases, decades. When necessary (such as during wound repair), these same cells can undergo rapid proliferation and turnover within a 5 day period (Folkman, J. and Shing, Y., *The Journal of Biological Chemistry*, **267**(16), 10931-10934, and Folkman, J. and Klagsbrun, M., *Science*, **235**, 442-447 (1987)).

Although angiogenesis is a highly regulated process under normal conditions, many diseases (characterized as angiogenic diseases) are driven by persistent unregulated angiogenesis. Otherwise stated, unregulated angiogenesis may either cause a particular disease directly or exacerbate an existing pathological condition. For example, ocular neovascularization has been implicated as the most common cause of blindness and dominates approximately 20 eye diseases. In certain existing conditions, such as arthritis, newly formed capillary blood vessels invade the joints and destroy cartilage. In diabetes, new capillaries formed in the retina invade the vitreous, bleed, and cause blindness. Growth and metastasis of solid tumors are also dependent on angiogenesis (Folkman, J., *Cancer Research*, **46**, 467-473 (1986), Folkman, J., *Journal of the National Cancer Institute*, **82**,

4-6 (1989). It has been shown, for example, that tumors which enlarge to greater than 2 mm must obtain their own blood supply and do so by inducing the growth of new capillary blood vessels. Once these new blood vessels become embedded in the tumor, they provide a means for tumor cells to enter the circulation and metastasize to distant sites such as liver, lung or bone (Weidner, N., *et al.*, *The New England Journal of Medicine*, **324**(1): 1-8 (1991)).

To date, several naturally occurring angiogenic factors have been described and characterized (Fidler, J.I. and Ellis, L.M., *Cell*, **79**: 185-189 (1994)). Recently, O'Reilly, *et al.* have isolated and purified a 38 kilodalton (kDa) protein from serum and urine of tumor-bearing mice that inhibits endothelial cell proliferation (O'Reilly, M. *et al.*, *Cell*, **79**: 315-328 (1994) and International Application WO 95/29242, published November 2, 1995). Microsequence analysis of this endothelial inhibitor showed 98% sequence homology to an internal fragment of murine plasminogen. Angiostatin, as the murine inhibitory fragment was named, was a peptide which included the first four kringle regions of murine plasminogen. A peptide fragment from the same region of human plasminogen (i.e. containing kringles 1-4) also strongly inhibited proliferation of capillary endothelial cells *in vitro* and *in vivo*. The intact plasminogen from which this peptide fragment was derived did not possess as potent an inhibitory effect.

Several angiogenesis inhibitors are currently under development for use in treating angiogenic diseases (Gasparini, G. and Harris, A. L., *J. Clin. Oncol.*, **13**(3): 765-782, (1995)), but there are disadvantages associated with these compounds. Suramin, for example, is a potent angiogenesis inhibitor but causes severe systemic toxicity in humans at doses required for antitumor activity. Compounds such as retinoids, interferons and antiestrogens are safe for human use but have weak antiangiogenic effects. Still other compounds may be difficult or costly to make.

Thus, there is a need for compounds useful in treating angiogenic diseases in mammals. More specifically, there is a need for angiogenesis inhibitors which are safe for therapeutic use and which exhibit selective toxicity with respect to the pathological condition such as by selectively inhibiting the proliferation of endothelial cells while exhibiting no or a low degree of toxicity to normal (ie. non-cancerous) cells. Such compounds should also be easily and cost-effectively made.

#### Summary of The Invention

In its principle embodiment, the present invention provides a kringle 5 peptide compound represented by the structural formula A-B-C-X-Y (I) or a pharmaceutically acceptable salt, ester or prodrug thereof, wherein A is absent or a nitrogen protecting group; Y is absent or a carboxylic acid protecting group; B is absent or is from 1 to about 197

naturally-occurring amino acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 530 of SEQ ID NO:1; C is R<sup>1</sup>-R<sup>2</sup>-R<sup>3</sup>-R<sup>4</sup> wherein R<sup>1</sup> is lysyl; R<sup>2</sup> is leucyl or arginyl; R<sup>3</sup> is tyrosyl, 3-I-tyrosyl or phenylalanyl; R<sup>4</sup> is aspartyl; and X is absent or is from 1 to about 12 naturally-occurring amino acid residues  
 5 corresponding to the sequence from amino acid position 535 to about amino acid position 546 of SEQ ID NO:1 and homologues and analogues thereof.

The present invention also includes a kringle 5 peptide compound represented by the structural formula A-B<sub>1</sub>-C<sub>1</sub>-X<sub>1</sub>-Y (II) or a pharmaceutically acceptable salt, ester or prodrug thereof wherein A is absent or a nitrogen protecting group; Y is absent or a  
 10 carboxylic acid protecting group; B<sub>1</sub> is absent or is from 1 to about 176 naturally-occurring amino acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 513 of SEQ ID NO:1; C<sub>1</sub> is the sequence from amino acid position 514 to amino acid position 523 of SEQ ID NO:1; and  
 15 X<sub>1</sub> is absent or is from 1 to about 10 naturally-occurring amino acid residues corresponding to the sequence from amino acid position 524 to amino acid position 533 of SEQ ID NO:1 and homologues and analogues thereof.

The present invention also includes a method for treating a patient in need of antiangiogenesis therapy comprising administering to the patient a compound containing a kringle 5 peptide fragment or kringle 5 fusion protein.

20 The present invention also includes a composition for treating a patient in need of anti-angiogenesis therapy comprising a compound containing a kringle 5 peptide fragment or kringle 5 fusion protein, kringle 5 antisera, kringle 5 receptor agonists and antagonists and kringle 5 antagonists linked to cytotoxic agents either alone or in combination with a pharmaceutically acceptable excipient and/or optionally sustained release compounds to form  
 25 a therapeutic composition.

The present invention also includes a composition for the treatment of a disease selected from the group consisting of cancer, arthritis, macular degeneration and diabetic retinopathy comprising a compound containing a kringle 5 peptide fragment or kringle 5 fusion protein.

30 The present invention also includes a composition comprising an isolated single or double-stranded polynucleotide sequence that encodes a kringle 5 peptide fragment or fusion protein. Such a polynucleotide is preferably a DNA molecule. The present invention also includes a vector containing a DNA sequence encoding a kringle 5 peptide fragment or fusion protein wherein the vector is capable of expressing a kringle 5 peptide fragment or  
 35 kringle 5 fusion protein when present in a cell and a composition comprising a cell containing a vector wherein the vector contains a DNA sequence encoding a kringle 5 peptide fragment or kringle 5 fusion protein. The present invention further encompasses

gene therapy methods whereby DNA sequences encoding a kringle 5 peptide fragment or kringle 5 fusion protein or kringle 5 peptide fragment conjugate are introduced into a patient to modify *in vivo* kringle 5 levels.

5 The present invention also includes a method of making a kringle 5 peptide fragment comprising the steps of: (a) exposing mammalian plasminogen to human or porcine elastase at a ratio of about 1:100 to about 1:300 to form a mixture of said plasminogen and said elastase; (b) incubating said mixture and (c) isolating the kringle 5 peptide fragment from said mixture.

10 The present invention also includes a method of making a kringle 5 peptide fragment comprising the steps of: (a) exposing mammalian plasminogen to human or porcine elastase at an elastase:plasminogen ratio of about 1:100 to about 1:300 to form a mixture of said elastase and said plasminogen; (b) incubating said mixture; and (c) isolating a protein conjugate of a kringle 5 peptide fragment from said mixture; (d) exposing said protein conjugate of the kringle 5 peptide fragment to pepsin at a ratio of about 1:0.2 to form a  
15 mixture of said pepsin and said plasminogen and (d) isolating said kringle 5 peptide fragment from said mixture. Alternatively, a kringle 5 peptide fragment or kringle 5 fusion protein can be made by a method comprising the steps of: (a) isolating a polynucleotide which encodes said kringle 5 peptide fragment or kringle 5 fusion protein; (b) cloning the polynucleotide into an expression vector; (c) transforming the vector into a suitable host cell; and growing the host cell under conditions suitable for the expression of the soluble  
20 kringle 5 peptide fragment or kringle 5 fusion protein.

#### Brief Description of the Figures

25 FIG. 1 shows the amino acid sequence of human plasminogen (SEQ ID NO: 1).

FIG. 2 shows the comparative homology in amino acid sequences of human (SEQ ID NO:34), mouse (SEQ ID NO:35), Rhesus monkey (SEQ ID NO:36), bovine (SEQ ID NO:37), and porcine (SEQ ID NO:38) kringle 5.

FIG. 3 shows the DNA sequence (SEQ ID NO: 12) of human plasminogen.

30 FIG. 4 shows a graph of the anti-proliferative activity of a single dose of various kringle fragments on bovine capillary endothelial (BCE) cells when tested in an *in vitro* cell proliferation assay.

FIG. 5 shows a map of expression vector pHil-D8 containing a leader sequence for recombinant protein secretion.

35 FIG. 6 shows a scan of a photograph of a Coomassie blue stained SDS-PAGE gel of culture supernatants (10  $\mu$ L/lane) of *Pichia pastoris* expressing a kringle 5 peptide fragment or fusion protein. Lanes 1, 6 and 10: negative controls; lanes 2, 3, and 4: three

distinct clones expressing K5A; lane 5: a clone expressing K5F; lanes 7 and 8: clones expressing K4-5A; lane 9: a clone expressing K4-5F. Arrows indicate protein bands of K5A (approximately 11 kDa) and K4-5F (approximately 20 kDa). Molecular weight markers are shown in the lanes preceding lanes 1 and 10.

FIG. 7 shows a scanned Coomassie blue stained SDS-PAGE gel of *E. coli* strains expressing a kringle 5 peptide fragment or fusion protein. Unless otherwise indicated, each lane contains 10  $\mu$ L of culture material equivalent to an  $A_{600}$  of 10. Lane 1: Low molecular weight markers; lane 2: K5A/pET32a, total culture; lane 3: K5A/pET32a, total culture (1/10 amount of lane 2); lane 4: K5A/pET32a, soluble fraction; lane 5: K5A/pET32a, insoluble fraction; lane 6: K4-5A/pET32a, total culture; lane 7: K4-5A/pET32a, total culture (1/10 amount of lane 6); lane 8: K4-5A/pET32a, soluble fraction; lane 9: K4-5A/pET32a, insoluble fraction; lane 10: K4-5A/pGEX-4T-2, total culture; lane 11: K4-5A/pGEX-4T-2, soluble fraction; lane 12: K4-5A/pGEX-4T-2, insoluble fraction; lane 13: kringle 5 standard; lane 14: high molecular weight markers.

#### Detailed Description of the Invention

As used herein, the term “kringle 5” (K5, hereinafter) refers to the region of mammalian plasminogen having three disulfide bonds which contribute to the specific three-dimensional confirmation defined by the fifth kringle region of the mammalian plasminogen molecule. One such disulfide bond links the cysteine residues located at amino acid positions 462 and 541, a second links the cysteine residues located at amino acid positions 483 and 524 and a third links the cysteine residues located at amino acid positions 512 and 536. The amino acid sequence of a complete mammalian plasminogen molecule (the human plasminogen molecule), including its kringle 5 region, is shown in FIG. 1 (SEQ ID NO: 1).

As used herein, the term “kringle 5 peptide fragment” refers to a peptide of between 4 and 104 amino acids (inclusive) with a substantial sequence homology to the corresponding peptide fragment of mammalian plasminogen, having an  $\alpha$ -N-terminus at about amino acid position 443 of intact mammalian plasminogen and an  $\alpha$ -C-terminus at about position 546. The total length of the a kringle 5 peptide fragment may vary depending upon the manner in which the kringle 5 peptide is obtained or may vary somewhat in sequence depending upon the species from which it is obtained. For example, certain forms of kringle 5 peptide fragments may be produced by proteolytic cleavage of glu-plasminogen, lys-plasminogen or miniplasminogen using the enzymes human or porcine elastase. When produced in this manner, the  $\alpha$ -C-terminal of the peptide resides at about amino acid 543 of SEQ ID NO: 1, but the  $\alpha$ -N-terminal amino acid may begin at amino acid position 443, 449 or 454. Thus, a kringle 5 peptide fragment resulting from human or porcine elastase digestion of glu-plasminogen, lys-plasminogen or miniplasminogen may have a total length

of either 101, 95 or 90 amino acids. A summary of these kringle 5 peptide fragments is shown in Table 1. When produced in the aforementioned manner, a pool of these three fragments is obtained wherein about 60% of the fragments have a length of 95 amino acids, about 35% of the fragments have the length of 101 amino acids and about 5% of the fragments have a length of 90 amino acids. If desired, these various fragments may be further purified by reverse phase HPLC, a technique well-known to those skilled in the art. Notwithstanding this variation in length, a K5 peptide fragment of the present invention includes either the sequence Lys-Leu-Tyr-Asp (i.e. from amino acid position 531 to amino acid position 534 of SEQ ID NO:1) or Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp (i.e. from amino acid position 514 to amino acid position 523 of SEQ ID NO:1) or analogues thereof.

As used herein, the term "kringle 5 fusion protein" refers to a polypeptide comprising an amino acid sequence drawn from two or more individual proteins, one of which is a K5 peptide fragment. A fusion protein may be formed by the expression of a polynucleotide in which the coding sequence for a kringle 5 peptide fragment has been joined with the coding sequence of at least one other polypeptide such that the two (or more) reading frames are in frame. Preferred kringle 5 fusion proteins are those wherein kringle 5 peptide fragment is fused to a corresponding sequence of human plasminogen such as kringle 4 (K4), kringles 3-4 (K3-4), kringles 2-4 (K2-4) and kringles 1-4 (K1-4). A preferred K5 fusion protein is kringles 4-5 (K4-5). Other examples of kringle 5 fusion proteins of the present invention include a K5 peptide fragment or K4-5 further joined to a biological tag. Such fusion proteins may or may not be capable of being cleaved into the separate proteins from which they are derived.

As used herein, the term "conjugate of a K5 peptide fragment" means a kringle 5 peptide fragment chemically coupled to another protein to form a conjugate. Examples of conjugates of kringle 5 peptide fragments include a kringle 5 peptide fragment coupled to albumin or to a peptide fragment from another kringle region of mammalian plasminogen. Molecular weights of conjugates of kringle 5 peptide fragments are between about 1,000 and about 25,000 kDa.

As used herein, the term "substantial sequence homology" means approximately 60% amino acid identity, desirably at least approximately 70% amino acid identity, more desirably approximately 80% amino acid identity and most desirably approximately 95% amino acid identity of the corresponding peptide sequence of human plasminogen. Sequences having substantial sequence homology to human plasminogen are referred to as "homologues". In addition to having substantial sequence homology, homologues of the present invention demonstrate like biological activity (i.e. anti-angiogenesis activity) as K5 peptide fragments described herein. Because the amino acid sequence or the number of

amino acids in a kringle 5 peptide fragment may vary from species to species or from the method of production, the total number of amino acids in a kringle 5 peptide fragment cannot, in some instances, be defined exactly. Given that these sequences are identical in at least 73% of their amino acids, it is to be understood that the amino acid sequence of a kringle 5 peptide fragment is substantially similar among species and that methods of production of kringle 5 peptide fragments provide kringle 5 peptide fragments with substantial sequence homology to the corresponding amino acid sequences of human plasminogen. FIG. 2 shows the amino acid sequence of a human kringle 5 peptide fragment having 95 amino acids (SEQ ID NO:34) is in comparison with the sequences of kringle 5 fragments from murine (SEQ ID NO:35), Rhesus monkey (SEQ ID NO:36), bovine (SEQ ID NO:37) and porcine (SEQ ID NO:38) plasminogen.

The present invention also contemplates amino acid residue sequences that are analogous to sequences set forth herein such that those sequences (analogues) demonstrate like biological activity to disclosed kringle 5 peptide fragments and fusion proteins thereof. It is well known in the art that modifications and changes can be made without substantially altering the biological function of that peptide. In making such changes, substitutions of like amino acid residues can be made on the basis of relative similarity of side-chain substituents, for example, their size, charge, hydrophobicity, hydrophilicity and the like. Alterations of the type described may be made to enhance the peptide's potency or stability to enzymatic breakdown or pharmacokinetics. Thus, sequences deemed as within the scope of the invention, include those analogous sequences characterized by a change in amino acid residue sequence or type wherein the change does not alter the fundamental nature and biological activity of the aforementioned K5 peptide fragments and/or fusion proteins.

A K5 peptide fragment or K5 fusion protein of the present invention may be characterized on the basis of potency when tested for its ability to inhibit the growth of bovine capillary (BCE) cells *in vitro*. The data in Table 1 and FIG. 4 illustrate that the K5 peptide fragment having the sequence from amino acid position 443 to amino acid position 543 of SEQ ID NO:1 shows approximately a 300-fold increase in activity (i.e. at inhibiting BCE cell proliferation) when compared to the kringle 5 peptide fragment having the sequence from amino acid position 443 to amino acid position 546 of SEQ ID NO:1 and approximately an 800-fold increase in activity when compared to kringle 1-4 peptide fragments.

The term "isolated" as used herein means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, which is separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide

could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that the vector or composition is not part of its natural environment.

5 The term "primer" denotes a specific oligonucleotide sequence complementary to a target nucleotide sequence and used to hybridize to the target nucleotide sequence and serve as an initiation point for nucleotide polymerization catalyzed by either DNA polymerase, RNA polymerase or reverse transcriptase.

10 The term "probe" denotes a defined nucleic acid segment (or nucleotide analog segment, i.e., PNA) which can be used to identify specific DNA present in samples bearing the complementary sequence.

15 A "recombinant polypeptide" as used herein means at least a polypeptide which by virtue of its origin or manipulation is not associated with all or a portion of the polypeptide with which it is associated in nature and/or is linked to a polypeptide other than that to which it is linked in nature. A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence. It also may be generated in any manner, including chemical synthesis or expression of a recombinant expression system.

The term "synthetic peptide" as used herein means a polymeric form of amino acids of any length, which may be chemically synthesized by methods well-known to an ordinarily skill practitioner. These synthetic peptides are useful in various applications.

20 "Purified polynucleotide" refers to a polynucleotide of interest or fragment thereof which is essentially free, i.e., contains less than about 50%, preferably less than about 70%, and more preferably, less than about 90% of the protein with which the polynucleotide is naturally associated. Techniques for purifying polynucleotides of interest are well-known in the art and include, for example, disruption of the cell containing the polynucleotide with a chaotropic agent and separation of the polynucleotide(s) and proteins by ion-exchange chromatography, affinity chromatography and sedimentation according to density. Thus, "purified polypeptide" means a polypeptide of interest or fragment thereof which is essentially free, that is, contains less than about 50%, preferably less than about 70%, and more preferably, less than about 90% of cellular components with which the polypeptide of interest is naturally associated. Methods for purifying are known in the art.

30 "Polypeptide" as used herein indicates a molecular chain of amino acids and does not refer to a specific length of the product. Thus, peptides, oligopeptides and proteins are included within the definition of polypeptide. This term is also intended to refer to post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like.

35 "Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular



entities refer to cells which can be, or have been, used as recipients for recombinant vector or other transferred DNA, and include the original progeny of the original cell which has been transfected.

As used herein "replicon" means any genetic element, such as a plasmid, a chromosome or a virus, that behaves as an autonomous unit of polynucleotide replication within a cell.

A "vector" is a replicon in which another polynucleotide segment is attached, such as to bring about the replication and/or expression of the attached segment.

The term "control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, such control sequences generally include promoter, ribosomal binding site and terminators; in eukaryotes, such control sequences generally include promoters, terminators and, in some instances, enhancers. The term "control sequence" thus is intended to include at a minimum all components whose presence is necessary for expression, and also may include additional components whose presence is advantageous, for example, leader sequences.

"Operably linked" refers to a situation wherein the components described are in a relationship permitting them to function in their intended manner. Thus, for example, a control sequence "operably linked" to a coding sequence is ligated in such a manner that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The term "open reading frame" or "ORF" refers to a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5' -terminus and a translation stop codon at the 3' -terminus. A coding sequence can include, but is not limited to, mRNA, cDNA, and recombinant polynucleotide sequences.

The term "transformation" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion. For example, direct uptake, transduction or f-mating are included. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

"Purified product" refers to a preparation of the product which has been isolated from the cellular constituents with which the product is normally associated, and from other types of cells which may be present in the sample of interest.

5 All peptide sequences are written according to the generally accepted convention whereby the  $\alpha$ -N-terminal amino acid residue is on the left and the  $\alpha$ -C-terminal is on the right. As used herein, the term " $\alpha$ -N-terminal" refers to the free alpha-amino group of an amino acid in a peptide, and the term " $\alpha$ -C-terminal" refers to the free alpha-carboxylic acid terminus of an amino acid in a peptide.

10 As used herein, the term "N-protecting group" refers to those groups intended to protect the  $\alpha$ -N-terminal of an amino acid or peptide or to otherwise protect the amino group of an amino acid or peptide against undesirable reactions during synthetic procedures. Commonly used N-protecting groups are disclosed in Greene, "Protective Groups In Organic Synthesis," (John Wiley & Sons, New York (1981)), which is hereby incorporated  
15 by reference. Additionally, protecting groups can be used as prodrugs which are readily cleaved *in vivo*, for example, by enzymatic hydrolysis, to release the biologically active parent. N-protecting groups comprise loweralkanoyl groups such as formyl, acetyl ("Ac"), propionyl, pivaloyl, t-butylacetyl and the like; other acyl groups include 2-chloroacetyl, 2-bromoacetyl, trifluoroacetyl, trichloroacetyl, phthalyl, o-nitrophenoxycarbonyl,  $\alpha$ -  
20 chlorobutyryl, benzoyl, 4-chlorobenzoyl, 4-bromobenzoyl, 4-nitrobenzoyl and the like; sulfonyl groups such as benzenesulfonyl, p-toluenesulfonyl and the like; carbamate forming groups such as benzyloxycarbonyl, p-chlorobenzyloxycarbonyl, p-methoxybenzyloxycarbonyl, p-nitrobenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, p-bromobenzyloxycarbonyl, 3,4-dimethoxybenzyloxycarbonyl, 3,5-  
25 dimethoxybenzyloxycarbonyl, 2,4-dimethoxybenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitro-4,5-dimethoxybenzyloxycarbonyl, 3,4,5-trimethoxybenzyloxycarbonyl, 1-(p-biphenyl)-1-methylethoxycarbonyl,  $\alpha,\alpha$ -dimethyl-3,5-dimethoxybenzyloxycarbonyl, benzhydryloxycarbonyl, t-butyloxycarbonyl, diisopropylmethoxycarbonyl, isopropylloxycarbonyl, ethoxycarbonyl, methoxycarbonyl, allyloxycarbonyl, 2,2,2,-  
30 trichloroethoxycarbonyl, phenoxycarbonyl, 4-nitrophenoxycarbonyl, fluorenyl-9-methoxycarbonyl, cyclopentylloxycarbonyl, adamantylloxycarbonyl, cyclohexylloxycarbonyl, phenylthiocarbonyl and the like; arylalkyl groups such as benzyl, triphenylmethyl, benzyloxymethyl, 9-fluorenylmethyloxycarbonyl (Fmoc) and the like and silyl groups such as trimethylsilyl and the like. Preferred N-protecting groups are formyl, acetyl, benzoyl,  
35 pivaloyl, t-butylacetyl, phenylsulfonyl, benzyl, t-butyloxycarbonyl (Boc) and benzyloxycarbonyl (Cbz). For example, lysine may be protected at the  $\alpha$ -N-terminal by an

acid labile group (e.g. Boc) and protected at the  $\epsilon$ -N-terminal by a base labile group (e.g. Fmoc) then deprotected selectively during synthesis.

As used herein, the term "carboxy protecting group" refers to a carboxylic acid protecting ester or amide group employed to block or protect the carboxylic acid functionality while the reactions involving other functional sites of the compound are performed. Carboxy protecting groups are disclosed in Greene, "Protective Groups in Organic Synthesis" pp. 152-186 (1981), which is hereby incorporated by reference. Additionally, a carboxy protecting group can be used as a prodrug whereby the carboxy protecting group can be readily cleaved *in vivo*, for example by enzymatic hydrolysis, to release the biologically active parent. Such carboxy protecting groups are well known to those skilled in the art, having been extensively used in the protection of carboxyl groups in the penicillin and cephalosporin fields as described in U.S. Pat. No. 3,840,556 and 3,719,667, the disclosures of which are hereby incorporated herein by reference. Representative carboxy protecting groups are C<sub>1</sub>-C<sub>8</sub> loweralkyl (e.g., methyl, ethyl or t-butyl and the like); arylalkyl such as phenethyl or benzyl and substituted derivatives thereof such as alkoxybenzyl or nitrobenzyl groups and the like; arylalkenyl such as phenylethenyl and the like; aryl and substituted derivatives thereof such as 5-indanyl and the like; dialkylaminoalkyl such as dimethylaminoethyl and the like; alkanoyloxyalkyl groups such as acetoxymethyl, butyryloxymethyl, valeryloxymethyl, isobutyryloxymethyl, isovaleryloxymethyl, 1-(propionyloxy)-1-ethyl, 1-(pivaloyloxy)-1-ethyl, 1-methyl-1-(propionyloxy)-1-ethyl, pivaloyloxymethyl, propionyloxymethyl and the like; cycloalkanoyloxyalkyl groups such as cyclopropylcarbonyloxymethyl, cyclobutylcarbonyloxymethyl, cyclopentylcarbonyloxymethyl, cyclohexylcarbonyloxymethyl and the like; aroyloxyalkyl such as benzoyloxymethyl, benzoyloxyethyl and the like; arylalkylcarbonyloxyalkyl such as benzylcarbonyloxymethyl, 2-benzylcarbonyloxyethyl and the like; alkoxycarbonylalkyl or cycloalkyloxycarbonylalkyl such as methoxycarbonylmethyl, cyclohexyloxycarbonylmethyl, 1-methoxycarbonyl-1-ethyl and the like; alkoxycarbonyloxyalkyl or cycloalkyloxycarbonyloxyalkyl such as methoxycarbonyloxymethyl, t-butyloxycarbonyloxymethyl, 1-ethoxycarbonyloxy-1-ethyl, 1-cyclohexyloxycarbonyloxy-1-ethyl and the like; aryloxycarbonyloxyalkyl such as 2-(phenoxycarbonyloxy)ethyl, 2-(5-indanyloxycarbonyloxy)ethyl and the like; alkoxyalkylcarbonyloxyalkyl such as 2-(1-methoxy-2-methylpropan-2-oyloxy)ethyl and the like; arylalkyloxycarbonyloxyalkyl such as 2-(benzyloxycarbonyloxy)ethyl and the like; arylalkenyloxycarbonyloxyalkyl such as 2-(3-phenylpropen-2-yloxycarbonyloxy)ethyl and the like; alkoxycarbonylaminoalkyl such as t-butyloxycarbonylaminomethyl and the like; alkylaminocarbonylaminoalkyl such as methylaminocarbonylaminomethyl and the like; alkanoylaminoalkyl such as acetylaminomethyl and the like; heterocycliccarbonyloxyalkyl

such as 4-methylpiperazinylcarbonyloxymethyl and the like; dialkylaminocarbonylalkyl such as dimethylaminocarbonylmethyl, diethylaminocarbonylmethyl and the like; (5-(loweralkyl)-2-oxo-1,3-dioxolen-4-yl)alkyl such as (5-t-butyl-2-oxo-1,3-dioxolen-4-yl)methyl and the like; and (5-phenyl-2-oxo-1,3-dioxolen-4-yl)alkyl such as (5-phenyl-2-oxo-1,3-dioxolen-4-yl)methyl and the like.

Representative amide carboxy protecting groups are aminocarbonyl and loweralkylaminocarbonyl groups.

Preferred carboxy-protected compounds of the invention are compounds wherein the protected carboxy group is a loweralkyl, cycloalkyl or arylalkyl ester, for example, methyl ester, ethyl ester, propyl ester, isopropyl ester, butyl ester, sec-butyl ester, isobutyl ester, amyl ester, isoamyl ester, octyl ester, cyclohexyl ester, phenylethyl ester and the like or an alkanoyloxyalkyl, cycloalkanoyloxyalkyl, aroyloxyalkyl or an arylalkylcarbonyloxyalkyl ester. Preferred amide carboxy protecting groups are loweralkylaminocarbonyl groups. For example, aspartic acid may be protected at the  $\alpha$ -C-terminal by an acid labile group (e.g. t-butyl) and protected at the  $\beta$ -C-terminal by a hydrogenation labile group (e.g. benzyl) then deprotected selectively during synthesis.

As used herein, the term "loweralkylaminocarbonyl" means a  $-C(O)NHR^{10}$  group which caps the  $\alpha$ -C-terminal of a synthetic, kringle 5 peptide fragment wherein  $R^{10}$  is  $C_1$ - $C_4$  alkyl.

As used herein, the term "aminocarbonyl" indicates a  $-C(O)NH_2$  group which caps the  $\alpha$ -C-terminal of a synthetic, kringle 5 peptide fragment.

As used herein, the term "prodrug" refers to compounds which are rapidly transformed *in vivo* to yield the parent compound, for example, by enzymatic hydrolysis in blood. A thorough discussion is provided in T. Higuchi and V. Stella, Prodrugs as Novel Delivery Systems, Vol. 14 of the A.C.S. Symposium Series and in Edward B. Roche, ed., Bioreversible Carriers in Drug Design, American Pharmaceutical Association and Pergamon Press, 1987, both of which are incorporated herein by reference.

As used herein, the term "pharmaceutically acceptable prodrug" refers to (1) those prodrugs of the compounds of the present invention which are, within the scope of sound medical judgement, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, commensurate with a suitable benefit-to-risk ratio and effective for their intended use and (2) zwitterionic forms, where possible, of the parent compound.

The term "activated ester derivative" as used herein refers to acid halides such as acid chlorides, and activated esters including, but not limited to, formic and acetic acid derived anhydrides, anhydrides derived from alkoxycarbonyl halides such as isobutyloxycarbonylchloride and the like, N-hydroxysuccinimide derived esters, N-

hydroxyphthalimide derived esters, N-hydroxybenzotriazole derived esters, N-hydroxy-5-norbornene-2,3-dicarboxamide derived esters, 2,4,5-trichlorophenol derived esters and the like.

As used herein, the term "antiangiogenesis activity" refers to the capability of a molecule to inhibit the growth of blood vessels.

As used herein, the term "endothelial inhibiting activity" refers to the capability of a molecule to inhibit angiogenesis in general and, for example, to inhibit the growth or migration of bovine capillary endothelial cells in culture in the presence of fibroblast growth factor or other known growth factors.

As used herein, the term "ED<sub>50</sub>" is an abbreviation for the dose of a kringle 5 peptide fragment or fusion protein which is effective to inhibit the growth of blood vessels or inhibit the growth of bovine capillary endothelial cells in culture in the presence of fibroblast growth factor or other known growth factors or inhibit the migration of endothelial cells by one-half of what the growth or migration would be in the absence of the inhibitor.

As used herein, for the most part, the names of naturally-occurring amino acids and aminoacyl residues used herein follow the naming conventions suggested by the IUPAC Commission on the Nomenclature of Organic Chemistry and the IUPAC-IUB Commission on Biochemical Nomenclature as set out in Nomenclature of  $\alpha$ -Amino Acids (Recommendations, 1974), Biochemistry, 14(2), (1975). Accordingly, the terms "Ala," "Arg," "Asn," "Asp," "Cys," "Gln," "Glu," "Gly," "His," "Ile," "Leu," "Lys," "Met," "Phe," "Pro," "Ser," "Thr," "Trp," "Tyr" and "Val" refer to the amino acids alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine and their corresponding aminoacyl residues in peptides in their L-, D- or D, L- forms. Where no specific configuration is indicated, one skilled in the art would understand that the stereochemistry of the  $\alpha$ -carbon of the amino acids and aminoacyl residues in peptides described in this specification and the appended claims is the naturally occurring or "L" configuration with the exception of the achiral molecule glycine and with the further exception of any amino acids which are achiral or otherwise designated as "D-."

As used herein, the term "3-I-Tyr" means a L-, D-, or D,L-tyrosyl residue wherein a hydrogen radical ortho to the phenolic hydroxyl is replaced by an iodide radical. The iodide radical may be radioactive or nonradioactive.

The present invention also contemplates amino acid residues with nonnaturally occurring side chain residues such as homophenylalanine, phenylglycine, norvaline, norleucine, ornithine, thiazoylalanine (2-, 4- and 5- substituted) and the like.

Thus, it is to be understood that the present invention is contemplated to encompass any derivatives of kringle 5 peptide fragments and kringle 5 fusion proteins which have antiangiogenic activity and includes the entire class of kringle 5 peptide fragments and fusion proteins described herein and homologues or analogues of those fragments and proteins.

5 Additionally, the invention is not dependent on the manner in which the kringle 5 peptide fragment or fusion protein is produced, i.e. by (1) proteolytic cleavage of an isolated mammalian plasminogen, (2) by expression of a recombinant molecule having a polynucleotide which encodes the amino acid sequence of a kringle 5 peptide fragment or fusion protein and (3) solid phase synthetic techniques known to those of ordinary skill in  
10 the art.

In one embodiment, the present invention provides peptides with the general structure B-C-X wherein B is a 88-mer peptide beginning at Val<sup>443</sup> and ending at Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and X is a 9-mer peptide beginning at Tyr<sup>535</sup> and ending at Ala <sup>543</sup>  
15 of SEQ ID NO:1.

In another embodiment, the present invention provides peptides with the general structure B-C-X wherein B is a 82-mer peptide beginning at Val<sup>449</sup> and ending at Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and X is a 9-mer peptide beginning at Tyr<sup>535</sup> and ending at Ala <sup>543</sup>  
20 of SEQ ID NO:1.

In yet another embodiment, the present invention provides peptides with the general structure B-C-X wherein B is a 77-mer peptide beginning at Val<sup>454</sup> and ending at Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and X is a 9-mer peptide beginning at Tyr<sup>535</sup> and ending at Ala <sup>543</sup>  
25 of SEQ ID No: 1

In yet another embodiment, the present invention provides peptides with the general structure B-C-X wherein B is a 88-mer peptide beginning at Val<sup>443</sup> and ending at Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and and X is a 12-mer peptide beginning at Tyr<sup>535</sup> and ending at Phe <sup>546</sup> of SEQ ID NO:1.  
30

In yet another embodiment, the present invention provides peptides with the general structure structure B-C-X wherein B is a 82-mer peptide beginning at Val<sup>449</sup> and ending at Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and X is a 12-mer peptide beginning at Tyr<sup>535</sup> and ending at Phe<sup>546</sup> of SEQ ID NO:1.  
35

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SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and X is a 12-mer peptide beginning at Tyr<sup>535</sup> and ending at Phe<sup>546</sup> of SEQ ID NO:1.

5 In yet another embodiment, the present invention provides peptides with the general structure B-C-X wherein B is a 176-mer peptide beginning at Val<sup>355</sup> and ending at Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and X is a 12-mer peptide beginning at Tyr<sup>535</sup> and ending at Ala<sup>543</sup> of SEQ ID NO:1.

10 In yet another embodiment, the present invention provides peptides with the general structure B-C-X wherein B is a 176-mer peptide beginning at Val<sup>355</sup> and ending at Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and X is a 12-mer peptide beginning at Tyr<sup>535</sup> and ending at Phe<sup>546</sup> of SEQ ID NO:1.

15 In yet another embodiment, the present invention provides peptides with the general structure A-C-Y wherein A is acetyl; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and Y is aminocarbonyl.

In yet another embodiment, the present invention provides peptides with the general structure A-C-X-Y wherein A is acetyl; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; X is tyrosyl; and Y is aminocarbonyl.

20 In yet another embodiment, the present invention provides peptides with the general structure A-B-C-Y wherein A is acetyl; B is a dipeptide beginning at amino acid position Pro<sup>529</sup> and ending at amino acid position Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and Y is aminocarbonyl.

25 In yet another embodiment, the present invention provides peptides with the general structure A-B-C-Y wherein A is acetyl; B is a dipeptide beginning at amino acid position Pro<sup>529</sup> and ending at amino acid position Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; and Y is aminocarbonyl.

30 In yet another embodiment, the present invention provides peptides with the general structure A-B-C-X-Y wherein A is acetyl; B is a hexapeptide beginning at amino acid position Tyr<sup>525</sup> and ending at amino acid position Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; X is tyrosyl; and Y is aminocarbonyl.

35 In yet another embodiment, the present invention provides peptides with the general structure A-B-C-X-Y wherein A is acetyl; B is arginyl; C is a 4-mer peptide wherein R<sup>1</sup> and

R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; X is tyrosyl and Y is aminocarbonyl.

In yet another embodiment, the present invention provides peptides with the general structure A-B-C-X-Y wherein A is acetyl, B is a dipeptide beginning at amino acid position Pro<sup>529</sup> and ending at amino acid position Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; X is 3-I-tyrosyl and Y is aminocarbonyl.

In yet another embodiment, the present invention provides peptides with the general structure A-B-C-X-Y wherein A is acetyl, B is a dipeptide beginning at amino acid position Pro<sup>529</sup> and ending at amino acid position Arg<sup>530</sup> of SEQ ID NO:1; C is a 4-mer peptide wherein R<sup>1</sup> and R<sup>4</sup> are as previously defined, R<sup>2</sup> is leucyl and R<sup>3</sup> is tyrosyl; X is tyrosyl and Y is aminocarbonyl.

In yet another embodiment, the present invention provides peptides with the general structure A-B<sub>1</sub>-C<sub>1</sub>-X<sub>1</sub>-Y wherein A is acetyl; B<sub>1</sub> and X<sub>1</sub> are absent, C<sub>1</sub> is a 10-mer peptide beginning at amino acid position Arg<sup>514</sup> and ending at amino acid position Trp<sup>523</sup> of SEQ ID NO:1 and Y is aminocarbonyl.

Representative compounds of the invention include compounds wherein A is acetyl and Y is aminocarbonyl and B-C-X is

- (a) the sequence from amino acid positions 355-543 of SEQ ID NO:1;
- (b) the sequence from amino acid positions 355-546 of SEQ ID NO:1;
- (c) the sequence from amino acid positions 443-543 of SEQ ID NO:1;
- (d) the sequence from amino acid positions 449-543 of SEQ ID NO:1;
- (e) the sequence from amino acid positions 454-543 of SEQ ID NO:1;
- (f) the sequence from amino acid positions 443-546 of SEQ ID NO:1;
- (g) the sequence from amino acid positions 449-546 of SEQ ID NO:1;
- (h) the sequence from amino acid positions 454-546 of SEQ ID NO:1;
- (i) the sequence from amino acid positions 525-535 of SEQ ID NO:1;
- (j) the sequence from amino acid positions 529-535 of SEQ ID NO:1; and
- (k) the sequence from amino acid positions 530-535 of SEQ ID NO:1.

Another representative compound is one wherein A is acetyl and Y is aminocarbonyl and B<sub>1</sub>-C<sub>1</sub>-X<sub>1</sub> is the sequence from amino acid positions 514-523 of SEQ ID NO:1.

K5 fragments or K5 fusion proteins may be obtained by expression of a recombinant molecule comprising a polynucleotide having a sequence which encodes a protein having a kringle 5 peptide fragment and then purifying the peptide product which is expressed (see Menhart, N., *et al.*, *Biochemistry*, 32: 8799-8806 (1993)). The DNA sequence of human plasminogen has been published (Browne, M.J. *et al.* *Fibrinolysis*, 5(4): 257-260 (1991))



and is shown in FIG. 3(a-b) (SEQ ID NO: 12). A polynucleotide sequence encoding kringle 5 begins at about nucleotide position 1421 of SEQ ID NO: 12 and ends at about nucleotide position 1723.

5 The gene encoding a K5 peptide fragment or K5 fusion protein may be isolated from cells or tissues that express high levels of human plasminogen or K5 fusion proteins by (1) isolating messenger RNA from the tissue or cells, (2) using reverse transcriptase to generate the corresponding DNA sequence and (3) using the polymerase chain reaction (PCR) with the appropriate primers to amplify the DNA sequence coding for the active K5 amino acid sequence or fusion protein thereof. Furthermore, a polynucleotide encoding a K5 peptide  
10 fragment or K5 fusion protein may be cloned into any commercially available expression vector (such as pBR322, pUC vectors and the like) or expression/purification vectors (such as a GST fusion vector (Pharmacia®, Piscataway, NJ)) and then expressed in a suitable procaryotic, viral or eucaryotic host. Purification may then be achieved by conventional means or, in the case of a commercial expression/purification system, in accordance with  
15 manufacturer's instructions.

A K5 peptide fragment or K5 fusion protein may also be synthesized by standard methods of solid phase chemistry known to those of ordinary skill in the art. For example kringle 5 peptide fragments may be synthesized by solid phase chemistry techniques following the procedures described by Steward and Young (Steward, J.M. and Young, J.D., *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Company, Rockford, IL,  
20 (1984) using an Applied Biosystem synthesizer. Similarly, multiple fragments may be synthesized then linked together to form larger fragments. These synthetic peptide fragments can also be made with amino acid substitutions at specific locations to test for anti-angiogenesis activity *in vitro* and *in vivo*. For solid phase peptide synthesis, a  
25 summary of the many techniques may be found in J.M. Stewart and J.D. Young, *Solid Phase Peptide Synthesis*, W.H. Freeman Co. (San Francisco), 1963 and J. Meienhofer, *Hormonal Proteins and Peptides*, vol. 2, p. 46, Academic Press (New York), 1973. For classical solution synthesis see G. Schroder and K. Lupke, *The Peptides*, Vol. 1, Academic Press (New York). In general, these methods comprise the sequential addition of one or  
30 more amino acids or suitably protected amino acids to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid is protected by a suitable protecting group. The protected or derivatized amino acid is then either attached to an inert solid support or utilized in solution by adding the next amino acid in the sequence having the complimentary (amino or carboxyl) group suitably protected and under conditions suitable  
35 for forming the amide linkage. The protecting group is then removed from this newly added amino acid residue and the next amino acid (suitably protected) is added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining

protecting groups (and any solid support) are removed sequentially or concurrently to afford the final polypeptide. By simple modification of this general procedure, it is possible to add more than one amino acid at a time to a growing chain, for example, by coupling (under conditions which do not racemize chiral centers) a protected tripeptide with a properly protected dipeptide to form, after deprotection, a pentapeptide.

A particularly preferred method of preparing compounds of the present invention involves solid phase peptide synthesis wherein the amino acid  $\alpha$ -N-terminal is protected by an acid or base sensitive group. Such protecting groups should have the properties of being stable to the conditions of peptide linkage formation while being readily removable without destruction of the growing peptide chain or racemization of any of the chiral centers contained therein. Suitable protecting groups are 9-fluorenylmethyloxycarbonyl (Fmoc), t-butyloxycarbonyl (Boc), benzyloxycarbonyl (Cbz), biphenylisopropylloxycarbonyl, t-amylloxycarbonyl, isobornyloxycarbonyl,  $\alpha,\alpha$ -dimethyl-3,5-dimethoxybenzyloxycarbonyl, o-nitrophenylsulfenyl, 2-cyano-t-butyloxycarbonyl, and the like. The 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group is particularly preferred for the synthesis of kringle 5 peptide fragments. Other preferred side chain protecting groups are, for side chain amino groups like lysine and arginine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (pmc), nitro, p-toluenesulfonyl, 4-methoxybenzene-sulfonyl, Cbz, Boc, and adamantyloxycarbonyl; for tyrosine, benzyl, o-bromobenzyloxy-carbonyl, 2,6-dichlorobenzyl, isopropyl, t-butyl (t-Bu), cyclohexyl, cyclophenyl and acetyl (Ac); for serine, t-butyl, benzyl and tetrahydropyranyl; for histidine, trityl, benzyl, Cbz, p-toluenesulfonyl and 2,4-dinitrophenyl; for tryptophan, formyl; for aspartic acid and glutamic acid, benzyl and t-butyl and for cysteine, triphenylmethyl (trityl). In the solid phase peptide synthesis method, the  $\alpha$ -C-terminal amino acid is attached to a suitable solid support or resin. Suitable solid supports useful for the above synthesis are those materials which are inert to the reagents and reaction conditions of the stepwise condensation-deprotection reactions, as well as being insoluble in the media used. The preferred solid support for synthesis of  $\alpha$ -C-terminal carboxy peptides is 4-hydroxymethylphenoxymethyl-copoly(styrene-1% divinylbenzene). The preferred solid support for  $\alpha$ -C-terminal amide peptides is the 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamidoethyl resin available from Applied Biosystems (Foster City, CA). The  $\alpha$ -C-terminal amino acid is coupled to the resin by means of N,N'-dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide (DIC) or O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU), with or without 4-dimethylaminopyridine (DMAP), 1-hydroxybenzotriazole (HOBT), benzotriazol-1-yloxy-tris(dimethylamino)phosphonium-hexafluorophosphate (BOP) or bis(2-oxo-3-oxazolidinyl)phosphine chloride (BOPCl), mediated coupling for from about 1 to about 24 hours at a temperature of between 10 ° and 50 °C in a solvent such as

dichloromethane or DMF. When the solid support is 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy- acetamidoethyl resin, the Fmoc group is cleaved with a secondary amine, preferably piperidine, prior to coupling with the  $\alpha$ -C-terminal amino acid as described above. The preferred method for coupling to the deprotected 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-acetamidoethyl resin is O-benzotriazol-1-yl-N,N,N',N'-tetramethyluroniumhexafluoro- phosphate (HBTU, 1 equiv.) and 1-hydroxybenzotriazole (HOBt, 1 equiv.) in DMF. The coupling of successive protected amino acids can be carried out in an automatic polypeptide synthesizer as is well known in the art. In a preferred embodiment, the  $\alpha$ -N-terminal in the amino acids of the growing peptide chain are protected with Fmoc. The removal of the Fmoc protecting group from the  $\alpha$ -N-terminal side of the growing peptide is accomplished by treatment with a secondary amine, preferably piperidine. Each protected amino acid is then introduced in about 3-fold molar excess, and the coupling is preferably carried out in DMF. The coupling agent is normally O-benzotriazol-1-yl-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU, 1 equiv.) and 1-hydroxybenzotriazole (HOBt, 1 equiv.). At the end of the solid phase synthesis, the polypeptide is removed from the resin and deprotected, either in successively or in a single operation. Removal of the polypeptide and deprotection can be accomplished in a single operation by treating the resin-bound polypeptide with a cleavage reagent comprising thianisole, water, ethanedithiol and trifluoroacetic acid. In cases wherein the  $\alpha$ -C-terminal of the polypeptide is an alkylamide, the resin is cleaved by aminolysis with an alkylamine. Alternatively, the peptide may be removed by transesterification, e.g. with methanol, followed by aminolysis or by direct transamidation. The protected peptide may be purified at this point or taken to the next step directly. The removal of the side chain protecting groups is accomplished using the cleavage cocktail described above. The fully deprotected peptide is purified by a sequence of chromatographic steps employing any or all of the following types: ion exchange on a weakly basic resin (acetate form); hydrophobic adsorption chromatography on underivatized polystyrene-divinylbenzene (for example, Amberlite XAD); silica gel adsorption chromatography; ion exchange chromatography on carboxymethylcellulose; partition chromatography, e.g. on Sephadex G-25, LH-20 or countercurrent distribution; high performance liquid chromatography (HPLC), especially reverse-phase HPLC on octyl- or octadecylsilyl-silica bonded phase column packing. Molecular weights of these kringle 5 peptide fragments are determined using Fast Atom Bombardment (FAB) Mass Spectroscopy. Solid phase kringle 5 peptide fragment synthesis is illustrated in Examples 1 to 12.

Depending on how they are produced, a K5 peptide fragment or K5 fusion protein may exist with or without the aforementioned disulfide bonds of the kringle 5 region of mammalian plasminogen or in the case of a fusion protein with other mammalian kringle

regions, with or without the disulfide bonds of those corresponding regions or may exist with disulfide bonds forming a tertiary structure which differs from the tertiary structure found in native mammalian plasminogen. Kringle 5 peptide fragments produced by enzymatic cleavage of Glu-, Lys- or miniplasminogen with elastase and /or pepsin (enzymes which cleave at sites removed from the cysteine linkages) will contain the native tertiary kringle 5 protein structure; kringle 5 peptide fragments prepared by solid phase peptide synthesis may or may not contain cystyl amino acyl residues and kringle 5 peptide fragments prepared by expression may contain disulfide bonds at different positions than those found in kringle 5 peptide fragments produced by enzymatic cleavage.

The compounds of the invention, including but not limited to those specified in the examples, possess anti-angiogenic activity. As angiogenesis inhibitors, such compounds are useful in the treatment of both primary and metastatic solid tumors and carcinomas of the breast; colon; rectum; lung; oropharynx; hypopharynx; esophagus; stomach; pancreas; liver; gallbladder; bile ducts; small intestine; urinary tract including kidney, bladder and urothelium; female genital tract including cervix, uterus, ovaries, choriocarcinoma and gestational trophoblastic disease; male genital tract including prostate, seminal vesicles, testes and germ cell tumors; endocrine glands including thyroid, adrenal, and pituitary; skin including hemangiomas, melanomas, sarcomas arising from bone or soft tissues and Kaposi's sarcoma; tumors of the brain, nerves, eyes, and meninges including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas and meningiomas; solid tumors arising from hematopoietic malignancies such as leukemias and including chloromas, plasmacytomas, plaques and tumors of mycosis fungoides and cutaneous T-cell lymphoma/leukemia; lymphomas including both Hodgkin's and non-Hodgkin's lymphomas; prophylaxis of autoimmune diseases including rheumatoid, immune and degenerative arthritis; ocular diseases including diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, retrolental fibroplasia, neovascular glaucoma, rubeosis, retinal neovascularization due to macular degeneration and hypoxia; abnormal neovascularization conditions of the eye; skin diseases including psoriasis; blood vessel diseases including hemangiomas and capillary proliferation within atherosclerotic plaques; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; wound granulation; diseases characterized by excessive or abnormal stimulation of endothelial cells including intestinal adhesions, Crohn's disease, atherosclerosis, scleroderma and hypertrophic scars (i.e. keloids) and diseases which have angiogenesis as a pathologic consequence including cat scratch disease (*Rochela minalia quintosa*) and ulcers (*Helicobacter pylori*). Another use is as a birth control agent which inhibits ovulation and establishment of the placenta.

The compounds of the present invention may also be useful for the prevention of metastases from the tumors described above either when used alone or in combination with radiotherapy and/or other chemotherapeutic treatments conventionally administered to patients for treating angiogenic diseases. For example, when used in the treatment of solid tumors, compounds of the present invention may be administered with chemotherapeutic agents such as alpha inteferon, COMP (cyclophosphamide, vincristine, methotrexate and prednisone), etoposide, mBACOD (methortrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone), PRO-MACE/MOPP (prednisone, methotrexate (w/leucovin rescue), doxorubicin, cyclophosphamide, taxol, etoposide/mechlorethamine, vincristine, prednisone and procarbazine), vincristine, vinblastine, angiostatin, TNP-470, pentosan polysulfate, platelet factor 4, angiostatin, LM-609, SU-101, CM-101, Techgalan, thalidomide, SP-PG and the like. Other chemotherapeutic agents include alkylating agents such as nitrogen mustards including mechlorethamine, melphan, chlorambucil, cyclophosphamide and ifosfamide; nitrosoureas including carmustine, lomustine, semustine and streptozocin; alkyl sulfonates including busulfan; triazines including dacarbazine; ethylenimines including thiotepa and hexamethylmelamine; folic acid analogs including methotrexate; pyrimidine analogues including 5-fluorouracil, cytosine arabinoside; purine analogs including 6-mercaptopurine and 6-thioguanine; antitumor antibiotics including actinomycin D; the anthracyclines including doxorubicin, bleomycin, mitomycin C and methramycin; hormones and hormone antagonists including tamoxifen and corticosteroids and miscellaneous agents including cisplatin and brequinar. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy and kringle 5 administration with subsequent kringle 5 administration to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor.

Cytotoxic agents such as ricin may be linked to kringle 5 peptide fragments and thereby provide a tool for destruction of cells that bind kringle 5. Peptides linked to cytotoxic agents may be infused in a manner designed to maximize delivery to the desired location. For example, ricin-linked high affinity kringle 5 fragments may be delivered via cannula directly into the target or into vessels supplying the target site. Such agents may also be delivered in a controlled manner through osmotic pumps coupled to infusion cannulae. A combination of kringle 5 antagonists may be co-applied with stimulators of angiogenesis to increase vascularization of tissue. Therapeutic regimens of this type could provide an effective means of destroying metastatic cancer.

The compounds of the present invention may be used in the form of pharmaceutically acceptable salts derived from inorganic or organic acids. By "pharmaceutically acceptable salt" is meant those salts which are, within the scope of sound

medical judgement, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well-known in the art. For example, S. M. Berge, *et al.* describe pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences*, **1977**, 66: 1 *et seq.*, which is hereby incorporated herein by reference. The salts may be prepared *in situ* during the final isolation and purification of the compounds of the invention or separately by reacting a free base function with a suitable organic acid. Representative acid addition salts include, but are not limited to acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate (isethionate), lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, phosphate, glutamate, bicarbonate, p-toluenesulfonate and undecanoate. Also, the basic nitrogen-containing groups can be quaternized with such agents as lower alkyl halides such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl and diamyl sulfates; long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides; arylalkyl halides like benzyl and phenethyl bromides and others. Water or oil-soluble or dispersible products are thereby obtained. Examples of acids which may be employed to form pharmaceutically acceptable acid addition salts include such inorganic acids as hydrochloric acid, hydrobromic acid, sulphuric acid and phosphoric acid and such organic acids as oxalic acid, maleic acid, succinic acid and citric acid.

Basic addition salts can be prepared *in situ* during the final isolation and purification of kringle 5 peptide fragments by reacting a carboxylic acid-containing moiety with a suitable base such as the hydroxide, carbonate or bicarbonate of a pharmaceutically acceptable metal cation or with ammonia or an organic primary, secondary or tertiary amine. Pharmaceutically acceptable salts include, but are not limited to, cations based on alkali metals or alkaline earth metals such as lithium, sodium, potassium, calcium, magnesium and aluminum salts and the like and nontoxic quaternary ammonia and amine cations including ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, diethylamine, ethylamine and the like. Other representative organic amines useful for the formation of base addition salts include ethylenediamine, ethanolamine, diethanolamine, piperidine, piperazine and the like. Preferred salts of the compounds of the invention include phosphate, tris and acetate.

Kringle 5 peptide fragments, kringle 5 antisera, kringle 5 receptor agonists, kringle 5 receptor antagonists or combinations thereof may be combined with pharmaceutically

acceptable sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions. A sustained-release matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid-base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. A sustained-release matrix is desirably chosen from biocompatible materials such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (copolymers of lactic acid and glycolic acid) polyanhydrides, poly(ortho)esters, polypeptides, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (co-polymers of lactic acid and glycolic acid).

Kringle 5 peptide fragments, kringle 5 fusion proteins, kringle 5 receptor agonists, kringle 5 receptor antagonists or combinations thereof may be combined with pharmaceutically acceptable excipients or carriers to form therapeutic compositions. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The compositions may be administered parenterally, sublingually, intracisternally, intravaginally, intraperitoneally, rectally, buccally or topically (as by powder, ointment, drops, transdermal patch or iontophoresis device).

The term "parenteral," as used herein, refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion. Pharmaceutical compositions for parenteral injection comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity may be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents such as paraben, chlorobutanol, phenol sorbic acid and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride and the like.

Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents, such as aluminum monostearate and gelatin, which delay absorption. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide, poly(orthoesters) and poly(anhydrides). Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues. The injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable media just prior to use.

Topical administration includes administration to the skin, mucosa and surfaces of the lung and eye. Compositions for topical administration, including those for inhalation, may be prepared as a dry powder which may be pressurized or non-pressurized. In non-pressurized powder compositions, the active ingredient in finely divided form may be used in admixture with a larger-sized pharmaceutically acceptable inert carrier comprising particles having a size, for example, of up to 100 micrometers in diameter. Suitable inert carriers include sugars such as lactose. Desirably, at least 95% by weight of the particles of the active ingredient have an effective particle size in the range of 0.01 to 10 micrometers. For topical administration to the eye, a compound of the invention is delivered in a pharmaceutically acceptable ophthalmic vehicle such that the compound is maintained in contact with the ocular surface for a sufficient time period to allow the compound to penetrate the corneal and internal regions of the eye, as, for example, the anterior chamber, posterior chamber, vitreous body, aqueous humor, vitreous humor, cornea, iris/ciliary, lens, choroid/retina and sclera. The pharmaceutically acceptable ophthalmic vehicle may, for example, be an ointment, vegetable oil or an encapsulating material. Alternatively, a compound of the invention may be injected directly into the vitreous and aqueous humor.

The composition may be pressurized and contain a compressed gas such as nitrogen or a liquified gas propellant. The liquified propellant medium and indeed the total composition is preferably such that the active ingredient does not dissolve therein to any substantial extent. The pressurized composition may also contain a surface active agent such as a liquid or solid non-ionic surface active agent or may be a solid anionic surface active agent. It is preferred to use the solid anionic surface active agent in the form of a sodium salt.

Compositions for rectal or vaginal administration are preferably suppositories which may be prepared by mixing the compounds of this invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which



are solids at room temperature but liquids at body temperature and therefore melt in the rectum or vaginal cavity and release the active compound.

Compounds of the present invention may also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form may contain, in addition to a compound of the present invention, stabilizers, preservatives, excipients and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art. See, for example, Prescott, Ed., Methods in Cell Biology, Volume XIV, Academic Press, New York, N.Y. (1976), p. 33 *et seq.*, which is hereby incorporated herein by reference.

When used in the above or other treatments, a therapeutically effective amount of one of the compounds of the present invention may be employed in pure form or, where such forms exist, in pharmaceutically acceptable salt form and with or without a pharmaceutically acceptable excipient. A "therapeutically effective amount" of the compound of the invention means a sufficient amount of the compound to treat an angiogenic disease (for example, to limit tumor growth or to slow or block tumor metastasis) at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. Total daily dose of kringle 5 peptide fragments or fusions proteins to be administered locally or systemically to a human or other mammal host in single or divided doses may be in amounts, for example, from 0.0001 to 200 mg/kg body weight daily and more usually 1 to 300 mg/kg body weight. If desired, the effective daily dose may be divided into multiple doses for purposes of administration. Consequently, single dose compositions may contain such amounts or submultiples thereof to make up the daily dose.

It will be understood that agents which can be combined with the compound of the present invention for the inhibition, treatment or prophylaxis of angiogenic diseases are not limited to those listed above, but include, in principle, any agents useful for the treatment or prophylaxis of angiogenic diseases.

5       The present invention also provides isolated polynucleotides which encode a mammalian kringle 5 peptide fragment or fusion protein having angiogenesis inhibiting activity. Such polynucleotides may be used for the expression of recombinant kringle 5 peptide fragments or in gene therapy (as described below).

10       A polynucleotide of the present invention may be in the form of mRNA or DNA. Polynucleotides in the form of DNA, cDNA, genomic DNA, and synthetic DNA are within the scope of the present invention. The DNA may be double-stranded or single-stranded, and if single-stranded may be the coding (sense) strand or non-coding (anti-sense) strand. A polynucleotide of the invention may be an unmodified form or include a modification such as methylation or capping.

15       The coding sequence which encodes the polypeptide may be identical to the coding sequence provided herein or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptide as the DNA provided herein. This polynucleotide may include only the coding sequence for the polypeptide, or the coding sequence for the polypeptide and additional coding sequence  
20       such as a leader or secretory sequence or a proprotein sequence, or the coding sequence for the polypeptide (and optionally additional coding sequence) and non-coding sequence, such as a non-coding sequence 5' and/or 3' of the coding sequence for the polypeptide.

25       In addition, the invention includes variant polynucleotides containing modifications such as polynucleotide deletions, substitutions or additions; and any polypeptide modification resulting from the variant polynucleotide sequence. A polynucleotide of the present invention also may have a coding sequence which is a naturally occurring allelic variant of the coding sequence provided herein.

30       In addition, the coding sequence for the polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the polypeptide. The polynucleotides may also encode for a proprotein which is the protein plus additional 5' amino acid residues. A protein having a prosequence is a  
35       proprotein and may in some cases be an inactive form of the protein. Once the prosequence is cleaved an active protein remains. Thus, the polynucleotide of the present invention may

encode for a protein, or for a protein having a prosequence or for a protein having both a presequence (leader sequence) and a prosequence.

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a GST tag supplied by a pGEX vector to provide for purification of the polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein. See, for example, I. Wilson, et al., Cell 37:767 (1984).

The polynucleotide may be generated in any manner, including but not limited to chemical synthesis, replication, reverse transcription or transcription, which is based on the information provided by the sequence of bases in the region(s) from which the polynucleotide is derived; as such, it may represent either a sense or an antisense orientation of the original polynucleotide. A preferred method of generating a polynucleotide is by the polymerase chain reaction described in U.S. Patents 4,683,195 and 4,683,202, which are incorporated herein by reference.

It is contemplated that polynucleotides will be considered to hybridize to the sequences provided herein if there is at least 50%, and preferably at least 70%, identity between the polynucleotide and the sequence.

The present invention also provides vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the present invention and methods for producing polypeptides of the present invention by recombinant techniques. Such methods comprise culturing the host cells under conditions suitable for the expression of the kringle 5 derived polynucleotide and recovering the kringle 5 derived polypeptide from the cell culture.

The polynucleotides of the present invention may be employed for producing a polypeptide by recombinant techniques. Thus, the polynucleotide sequence may be included in any one of a variety of expression vehicles, in particular vectors or plasmids for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other plasmid or vector may be used so long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into appropriate restriction endonuclease sites by procedures known in the art. Such procedures and others are deemed

to be within the scope of those skilled in the art. The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. Representative examples of such promoters include but are not limited to LTR or SV40 promoter, the *E. coli* lac or trp, the phage lambda P sub L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Salmonella typhimurium*; *Streptomyces spp.*; fungal cells, such as yeast; insect cells such as *Drosophila* and Sf9; and animal cells such as CHO, COS or Bowes, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings provided herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pSPORT1 (GIBCO BRL, Gaithersburg, MD), pQE70, pQE60, pQE-9 (Qiagen) pBs, phagescript, psiX174, pBluescript SK, pBsKS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene®, La Jolla, CA); pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia®). Eukaryotic: pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene®) pSVK3, pBPV, pMSG, pSVL (Pharmacia®). However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, SP6, T7, gpt, lambda P sub R, P sub L and trp. Eukaryotic promoters

include cytomegalovirus (CMV) immediate early, herpes simplex virus (HSV) thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention provides host cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (L. Davis et al., "Basic Methods in Molecular Biology", 2nd edition, Appleton and Lang, Paramount Publishing, East Norwalk, CT (1994)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor, N.Y., 1989), which is hereby incorporated by reference.

Transcription of a DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), alpha factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification

peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a routine matter of choice.

Useful expression vectors for bacterial use comprise a selectable marker and bacterial origin of replication derived from plasmids comprising genetic elements of the well-known cloning vector pBR322 (ATCC 37017). Other vectors include but are not limited to PKK223-3 (Pharmacia® Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Useful expression vectors may also comprise a fusion partner for ease in purifying a desired polypeptide of the invention or for producing soluble polypeptides. Examples of commercial fusion vectors include but are not limited to pET32a (Novagen, Madison, WI), pGEX-4T-2 (Pharmacia®) and pCYB3 (New England Biolabs, Beverly, MA). Expression vectors which avoid the use of fusion partners may also be constructed particularly for high level expression of kringle 5 peptide fragments or fusion proteins in bacterial cells. For example, vectors can be made to optimize for translational coupling as described by Pilot-Matias, T. J., *et al.*, in *Gene*, **128**: 219-225 (1993), incorporated herein by reference.

Alternatively, a polynucleotide of the invention may be co-expressed with a separate accessory plasmid which itself encodes a protein or peptide that aids in solubilizing the first peptide of interest (see, e.g. Makrides, S.C., *Microbiological Reviews*, **60**: 512 (1996)). For example, certain kringle 5 peptide fragments (which have been shown to be produced as soluble fusion proteins with thioredoxin (see Example 20)) may be expressed from a non-fusion vector simultaneously with (i.e. in the same host cell as) a second vector that expresses thioredoxin.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is derepressed by appropriate means (e.g., temperature shift or chemical induction), and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw

cycling, sonication, mechanical disruption, or use of cell lysing agents; such methods are well-known to the ordinary artisan.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts described by Gluzman, Cell 23:175 (1981), and other cell lines capable of expressing a compatible vector, such as the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements. Representative, useful vectors include pRc/CMV and pcDNA3 (available from Invitrogen, San Diego, CA).

The present invention also encompasses gene therapy whereby the gene encoding kringle 5 peptide fragments or kringle 5 peptide fragment conjugates is regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise referred to as gene therapy, are disclosed in Gene Transfer into Mammalian Somatic Cells *in vivo*, N. Yang, Crit. Rev. Biotechn. 12(4): 335-356 (1992), which is hereby incorporated herein by reference. Gene therapy encompasses incorporation of polynucleotide sequences into somatic cells or germ line cells for use in either *ex vivo* or *in vivo* therapy. Gene therapy functions to replace genes, to augment normal or abnormal gene function and to combat infectious diseases and other pathologies.

Strategies for treating medical problems with gene therapy include therapeutic strategies such as identifying the defective gene and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene or prophylactic strategies such as adding a gene which encodes a protein product that will treat the condition or that will make the tissue or organ more susceptible to a treatment regimen. As an example of a prophylactic strategy, a gene encoding a kringle 5 peptide fragment or a kringle 5 peptide fragment conjugate may be placed in a patient and thus prevent occurrence of angiogenesis or a gene that makes tumor cells more susceptible to radiation could be inserted so that radiation of the tumor would cause increased killing of the tumor cells.

Many protocols for the transfer of DNA encoding a kringle 5 peptide fragment or kringle 5 fusion protein or for transfer of the DNA for kringle 5 peptide fragment regulatory sequences (or those of the fusion partner) are envisioned in this invention. Transfection of promoter sequences, other than ones specifically associated with a kringle 5 peptide fragment or other sequences which would increase production of kringle 5 peptide fragments, are also envisioned as methods of gene therapy. An example of this technology

is found in Transkaryotic Therapies, Inc., of Cambridge, Massachusetts, using homologous recombination to insert a "genetic switch" which turns on an erythropoietin gene in cells as disclosed in Genetic Engineering News, April 15, 1994, which is hereby incorporated herein by reference. Such "genetic switches" could be used to activate a kringles 5 peptide fragment (or a kringles 5 receptor) in cells not normally expressing these proteins.

Gene transfer methods for gene therapy fall into three broad categories: (1) physical (e.g., electroporation, direct gene transfer and particle bombardment), (2) chemical (e.g. lipid-based carriers and other non-viral vectors) and (3) biological (e.g. virus derived vectors). For example, non-viral vectors such as liposomes coated with DNA may be directly injected intravenously into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA to macrophages and Kupffer cells. Vectors or the "naked" DNA of the gene may also be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include *ex vivo* gene transfer, *in vivo* gene transfer and *in vitro* gene transfer. In *ex vivo* gene transfer, cells are taken from the patient and grown in cell culture. The DNA is transfected into the cells, and the transfected cells are expanded in number and then reimplanted in the patient. In *in vitro* gene transfer, the transformed cells are cells growing in culture, such as tissue culture cells, and not particular cells from a particular patient. These "laboratory cells" are transfected, and the transfected cells are selected and expanded for either implantation into a patient or for other uses. *In vivo* gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. All three of the broad based categories described above may be used to achieve gene transfer *in vivo*, *ex vivo* and *in vitro*.

Mechanical (i.e. physical) methods of DNA delivery can be achieved by microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles such as the gold particles used in a "gene gun" and inorganic chemical approaches such as calcium phosphate transfection. It has been found that physical injection of plasmid DNA into muscle cells yields a high percentage of cells which are transfected and have a sustained expression of marker genes. The plasmid DNA may or may not integrate into the genome of the cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative tissues for a prolonged period of time without fear of mutational insertions, deletions or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily permanent, transfer of therapeutic genes into specific cells may provide treatments for genetic diseases or for prophylactic use. The DNA could be reinjected periodically to maintain the gene product level without mutations occurring in the genomes of the recipient cells. Non-integration of



exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

Particle-mediated gene transfer may also be employed for injecting DNA into cells, tissues and organs. With a particle bombardment device, or "gene gun," a motive force is generated to accelerate DNA-coated high density particles (such as gold or tungsten) to a high velocity that allows penetration of the target organs, tissues or cells. Electroporation for gene transfer uses an electrical current to make cells or tissues susceptible to electroporation-mediated gene transfer. A brief electric impulse with a given field strength is used to increase the permeability of a membrane in such a way that DNA molecules can penetrate into the cells. The techniques of particle-mediated gene transfer and electroporation are well known to those of ordinary skill in the art.

Chemical methods of gene therapy involve carrier-mediated gene transfer through the use of fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion. A carrier harboring a DNA of interest can be conveniently introduced into body fluids or the bloodstream and then site specifically directed to the target organ or tissue in the body. Cell or organ-specific DNA-carrying liposomes, for example, can be developed and the foreign DNA carried by the liposome absorbed by those specific cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing that receptor. Another carrier system that has been used is the asialoglycoprotein/polylysine conjugate system for carrying DNA to hepatocytes for *in vivo* gene transfer.

Transfected DNA may also be complexed with other kinds of carriers so that the DNA is carried to the recipient cell and then deposited in the cytoplasm or in the nucleoplasm. DNA can be coupled to carrier nuclear proteins in specifically engineered vesicle complexes and carried directly into the nucleus.

Carrier mediated gene transfer may also involve the use of lipid-based compounds which are not liposomes. For example, lipofectins and cytofectins are lipid-based positive ions that bind to negatively charged DNA and form a complex that can ferry the DNA across a cell membrane. Another method of carrier mediated gene transfer involves receptor-based endocytosis. In this method, a ligand (specific to a cell surface receptor) is made to form a complex with a gene of interest and then injected into the bloodstream. Target cells that have the cell surface receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

Biological gene therapy methodologies employ viral vectors or non-viral vectors (such as the ligand-DNA conjugates, liposomes and the lipid-DNA complexes discussed above) to insert genes into cells. The transfected cells may be cells derived from the patient's normal tissue, the patient's diseased tissue or non-patient cells.

It may be desirable that a recombinant DNA molecule comprising a kringle 5 peptide fragment DNA sequence or a kringle 5 fusion protein DNA sequence is operatively linked to an expression control sequence to form an expression vector capable of expressing a kringle 5 peptide fragment or fusion protein respectively. Alternatively, gene regulation of a kringle 5 peptide fragment or a kringle 5 fusion protein may be accomplished by administering compounds that bind to the kringle 5 gene, the fusion partner gene or control regions associated with the kringle 5 gene or the gene of its fusion partner or to a corresponding RNA transcript (of either) to modify the rate of transcription or translation.

Viral vectors that have been used for gene therapy protocols include, but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus, adenovirus, adeno-associated virus, herpes viruses, SV 40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors are the most widely utilized gene transfer vectors. Murine leukemia retroviruses are composed of a single strand RNA complexed with a nuclear core protein and polymerase (pol) enzymes encased by a protein core (gag) and surrounded by a glycoprotein envelope (env) that determines host range. The genomic structure of retroviruses include gag, pol, and env genes enclosed at the 5' and 3' long terminal repeats (LTRs). Retroviral vector systems exploit the fact that a minimal vector containing the 5' and 3' LTRs and the packaging signal are sufficient to allow vector packaging and infection and integration into target cells providing that the viral structural proteins are supplied *in trans* in the packaging cell line. Fundamental advantages of retroviral vectors for gene transfer include efficient infection and gene expression in most cell types, precise single copy vector integration into target cell chromosomal DNA and ease of manipulation of the retroviral genome. For example, altered retrovirus vectors have been used in *ex vivo* methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes or other somatic cells (which may then be introduced into the patient to provide the gene product from the inserted DNA).

The adenovirus is composed of linear, double stranded DNA complexed with core proteins and surrounded with capsid proteins. Advances in molecular virology have led to the ability to exploit the biology of these organisms to create vectors capable of transducing novel genetic sequences into target cells *in vivo*. Adenoviral-based vectors will express gene product peptides at high levels. Adenoviral vectors have high efficiencies of infectivity, even with low titers of virus. Additionally, the virus is fully infective as a cell-free virion so injection of producer cell lines are not necessary. Another potential advantage to adenoviral vectors is the ability to achieve long term expression of heterologous genes *in vivo*.

Viral vectors have also been used to insert genes into cells using *in vivo* protocols. To direct tissue-specific expression of foreign genes, cis-acting regulatory elements or

promoters that are known to be tissue-specific may be used. Alternatively, this can be achieved using *in situ* delivery of DNA or viral vectors to specific anatomical sites *in vivo*. For example, gene transfer to blood vessels *in vivo* was achieved by implanting *in vitro* transduced endothelial cells in chosen sites on arterial walls. The virus-infected surrounding cells, in turn, also expressed the gene product. A viral vector can be delivered directly to the *in vivo* site (by catheter, for example) thus allowing only certain areas to be infected by the virus and providing long-term, site-specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Kringle 5 peptide fragments may also be produced and used in a variety of applications. As examples, different peptide fragments of kringle 5 can be used (1) as agonists and antagonists active at kringle 5 binding sites, (2) as antigens for the development of specific antisera, (3) as peptides for use in diagnostic kits and (4) as peptides linked to or used in combination with cytotoxic agents for targeted killing of cells that bind kringle 5 peptide fragments. The amino acid sequences that comprise these peptide fragments may be selected on the basis of their position on the exterior regions of the molecule which are accessible for binding to antisera or the inhibitory potency of the peptide fragments toward processes arising from or exasperated by angiogenesis. Furthermore, these peptide sequences may be compared to known sequences using protein sequence databases such as GenBank, Brookhaven Protein, SWISS-PROT, and PIR to determine potential sequence homologies. This information facilitates elimination of sequences that exhibit a high degree of sequence homology to other molecules and thereby enhances the potential for high specificity in the development of antisera, agonists and antagonists to kringle 5.

Kringle 5 peptide fragments or fusion proteins may also be used as a means to isolate a kringle 5 receptor by immobilization of the kringle 5 peptide fragment or fusion protein on a solid support in, for example, an affinity column through which cultured endothelial cells or membrane extracts are passed. As is known in the art, isolation and purification of a kringle 5 receptor may be followed by amino acid sequencing to identify and isolate polynucleotides which encode the kringle 5 receptor. Such polynucleotides may then be cloned into a suitable expression vector and transfected into tumor cells. Expression of the receptor by the transfected tumor cells would enhance the responsiveness of these cells to endogenous or exogenous kringle 5 peptide fragments and thereby decrease the rate of metastatic growth. Furthermore, recombinant expression of this receptor would allow greater amounts of receptor to be produced, e.g. to produce a sufficient quantity for use in high throughput screening assays to identify smaller antagonists which mimic the action of kringle 5.

Systematic substitution of amino acids within these synthesized peptides may yield high affinity peptide agonists and antagonists to the kringle 5 receptor that enhance or diminish kringle 5 peptide fragment binding to its receptor. Such agonists may be used to suppress the growth of micrometastases and thereby limit the spread of cancer. In cases of inadequate vascularization, antagonists to kringle 5 peptide fragments may be applied to block the inhibitory effects of kringle 5 peptide fragments and promote angiogenesis. For example, this type of treatment may have therapeutic effects in promoting wound healing in diabetics.

Kringle 5 peptide fragments or fusion proteins or conjugates of the present invention can also be used as antigens to generate polyclonal or monoclonal antibodies which are specific for the kringle 5 inhibitor. One way in which such antibodies could be used is in diagnostic methods and kits to detect or quantify kringle 5 peptide fragments in a body fluid or tissue. Results from these tests could be used to diagnose or determine the prognostic relevance of kringle 5 peptide fragments.

Kringle 5 peptide fragments or kringle 5 fusion proteins may be labeled with radioactive isotopes (See Example 13) or chemically coupled to proteins to form conjugates. Conjugates include enzymes, carrier proteins, cytotoxic agents, fluorescent, chemiluminescent and bioluminescent molecules which are used to facilitate the testing of the ability of compounds containing kringle 5 peptide fragments to bind kringle 5 antisera, detect cell types which possess a kringle 5 peptide fragment receptor or aid in purification of kringle 5 peptide fragments. The coupling technique is generally chosen on the basis of the functional groups available on the amino acids of the kringle 5 peptide fragment sequence including, but not limited to alkyl, amino, sulfhydryl, carboxyl, amide, phenol, indolyl and imidazolyl. Various reagents used to effect such couplings include, among others, glutaraldehyde, diazotized benzidine, carbodiimides and p-benzoquinone. The efficiency of the coupling reaction is determined using different techniques appropriate for the specific reaction. For example, radiolabeling of a kringle 5 peptide or a biologically active fragment thereof with  $I^{125}$  may be accomplished using chloramine T and  $NaI^{125}$  of high specific activity. The reaction is terminated with sodium metabisulfite and the mixture is desalted on disposable columns. The labeled peptide is eluted from the column and the fractions are collected. Aliquots are removed from each fraction and radioactivity is measured in a gamma counter. This procedure provides the radiolabeled kringle 5 peptide fragment free from unreacted  $NaI^{125}$ . In another example, blood or tissue extracts containing a kringle 5 peptide fragment coupled to kringle 4 may be purified on a polylysine resin affinity column whereby the kringle 4-kringle 5 peptide fragment binds to the resin through the affinity of the kringle 4 peptide fragment for lysine. Elution of the bound protein would provide a purified kringle 4-kringle 5 peptide fragment.

Another application of peptide conjugation is the production of polyclonal antisera. The production of antiserum against kringle 5 peptide fragments, kringle 5 peptide fragment analogs and the kringle 5 receptor can be performed using established techniques known to those skilled in the art. For example, kringle 5 peptide fragments containing lysine residues may be linked to purified bovine serum albumin (BSA) using glutaraldehyde. The efficiency of this reaction may be determined by measuring the incorporation of radiolabeled peptide. Unreacted glutaraldehyde and peptide may be separated by dialysis, and the conjugate may be used to raise polyclonal antisera in rabbits, sheep, goats or other animals. Kringle 5 peptide fragments conjugated to a carrier molecule such as BSA may be combined with an adjuvant mixture, emulsified and injected subcutaneously at multiple sites on the back, neck, flanks, and sometimes in the footpads of a suitable host. Generally, booster injections are then given at regular intervals, such as every 2 to 4 weeks. Approximately 7 to 10 days after each injection, blood samples are obtained by venipuncture using, for example, the marginal ear veins after dilation. The blood samples are allowed to clot overnight at 4°C and are centrifuged at approximately 2400 X g at 4°C for about 30 minutes. The serum is removed, aliquoted and stored at 4°C for immediate use or at -20 to -90°C for subsequent analysis.

Serum samples from generation of polyclonal antisera or media samples from production of monoclonal antisera may be analyzed for determination of antibody titer and, in particular, for the determination of high titer antisera. Subsequently, the highest titer kringle 5 peptide fragment antisera may be tested to establish the following: a) optimal antiserum dilution for highest specific binding of the antigen and lowest non-specific binding, b) ability to bind increasing amounts of kringle 5 peptide fragments in a standard displacement curve, c) potential cross-reactivity with related peptides and proteins including plasminogen and kringle 5 peptide fragments of related species and d) ability to detect kringle 5 peptide fragments in cell culture media and in extracts of plasma, urine and tissues. Titer may be established through several means known in the art, such as by dot blot and density analysis and also by precipitation of radiolabeled peptide-antibody complexes using protein A, secondary antisera, cold ethanol or charcoal-dextran followed by activity measurement with a gamma counter. If desired, the highest titer antisera may be purified on affinity columns. For example, kringle 5 peptide fragments may be coupled to a commercially available resin and used to form an affinity column. Antiserum samples may then be passed through the column so that kringle 5 antibodies bind (via kringle 5 peptide fragments) to the column. These bound antibodies are subsequently eluted, collected and evaluated for determination of titer and specificity.

Kits for measurement of kringle 5 peptide fragments and the kringle 5 receptor are also contemplated as part of the present invention. Antisera that possess the highest titer and

specificity and can detect kringle 5 peptide fragments in extracts of plasma, urine, tissues and cell culture media may be used to establish assay kits for rapid, reliable, sensitive and specific measurement and localization of kringle 5 peptide fragments. These assay kits may employ, but are not limited to, the following techniques: competitive and non-competitive assays, radioimmunoassays, bioluminescence and chemiluminescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISAs, microtiter plates, immunocytochemistry and antibody-coated strips or dipsticks for rapid monitoring of urine or blood. For each kit the range, sensitivity, precision, reliability, specificity and reproducibility of the assay are established by means well known to those skilled in the art.

One example of an assay kit commonly used in research and in the clinic is a radioimmunoassay (RIA) kit. A kringle 5 peptide fragment RIA may be established in the following manner: After successful radioiodination and purification of a kringle 5 peptide fragment, antiserum possessing the highest titer of anti-kringle 5 peptide fragment antibodies is added at several dilutions to tubes containing a relatively constant amount of radioactivity, such as 10,000 cpm, in a suitable buffer system. (Buffer or preimmune serum is added to other tubes to determine non-specific binding). After incubation at 4°C for 24 hours, protein A is added to all tubes and the tubes are vortexed, incubated at room temperature for 90 minutes and centrifuged at approximately 2000 - 2500 X g at 4°C to precipitate the complexes of antibody bound to labeled antigen. The supernatant is removed by aspiration and radioactivity in the pellets counted in a gamma counter. The antiserum dilution that binds approximately 10 to 40% of the labeled peptide after subtraction of the non-specific binding is selected for further characterization.

Next, a dilution range (approximately 0.1 pg to 10 ng) of the kringle 5 peptide fragment used for development of the antiserum is evaluated by adding known amounts of the peptide to tubes containing radiolabeled peptide and antiserum. After an incubation period (24 or 48 hours, for example), protein A is added and the tubes are centrifuged, the supernatant is removed and the radioactivity in the pellet is counted. The displacement of the binding of radiolabeled the kringle 5 peptide fragment by the unlabeled kringle 5 peptide fragment (standard) provides a standard curve. Additionally, several concentrations of other kringle 5 peptide fragments, plasminogens, kringle 5 peptide fragments from different species and homologous peptides may be added to the assay tubes to characterize the specificity of the kringle 5 peptide fragment antiserum.

Thereafter, extracts of various tissues including, but not limited to, primary and secondary tumors, Lewis lung carcinoma, cultures of kringle 5 peptide fragment-producing cells, placenta, uterus and other tissues such as brain, liver and intestine are prepared using extraction techniques that have been successfully employed to extract kringle 5 peptide

fragments. After workup of the tissue extracts, assay buffer is added and different aliquots are placed into the RIA tubes. Extracts of known kringle 5 peptide fragment-producing cells produce displacement curves that are parallel to the standard curve whereas extracts of tissues that do not produce kringle 5 peptide fragments do not displace radiolabeled kringle 5 peptide fragments from the kringle 5 peptide fragment antiserum. Such displacement curves indicate the utility of the kringle 5 peptide fragment assay to measure kringle 5 peptide fragments in tissues and body fluids.

Tissue extracts that contain kringle 5 peptide fragments may also be characterized by subjecting aliquots to reverse phase HPLC. Eluate fractions are collected, dried in Speed Vac, reconstituted in RIA buffer and analyzed in the kringle 5 RIA. In this case, the maximal amount of kringle 5 peptide fragment immunoreactivity is located in the fractions corresponding to the elution position of the kringle 5 peptide fragment.

The above described assay kit would provide instructions, antiserum, a kringle 5 peptide fragment and possibly a radiolabeled kringle 5 peptide fragment and/or reagents for precipitation of bound kringle 5 peptide fragment/kringle 5 antibody complexes. Such a kit would be useful for the measurement of kringle 5 peptide fragments in biological fluids and tissue extracts of animals and humans with and without tumors.

Another kit may be used to visualize or localize kringle 5 peptide fragments in tissues and cells. For example, immunohistochemistry techniques and kits which employ such techniques are well known to those of ordinary skill in the art. As is known in the art, an immunohistochemistry kit would provide kringle 5 peptide fragment antiserum, and possibly blocking serum and secondary antiserum linked to a fluorescent molecule such as fluorescein isothiocyanate or to some other reagent used to visualize the primary antiserum. Using this methodology, biopsied tumors may be examined for sites of kringle 5 peptide fragment production or for sites of the kringle 5 peptide fragment receptor. Alternatively, a kit may supply radiolabeled nucleic acids for use in *in situ* hybridization to probe for kringle 5 peptide fragment messenger RNA.

The compounds of the invention may be prepared using processes well known to those of ordinary skill in the art. (See for example, Sottrup-Jensen *et al.*, Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3, Davidson, J.F., Rowan, R.M., Samama, M.M. and Desnoyers, P.C. editors, Raven Press, New York, 1978. One manner of preparing kringle 5 peptide fragments is by enzymatic cleavage of the native protein (glu-plasminogen) or a variant thereof (meaning a truncated form of the full length protein which is amenable to cleavage by enzymatic digestion and which comprises at least a kringle 5 sequence as defined above such as lys-plasminogen or miniplasminogen). This method first requires isolating the protein from human plasma in the absence of plasmin inhibitors and thereby promoting the conversion of glu-plasminogen to lys-plasminogen (see Novokhatny,

V and Kudinov, S.A., J. Mol. Biol. 179: 215-232 (1984). Subsequently, the truncated molecule is treated with an proteolytic enzyme at a concentration sufficient to cleave kringle 5 peptide fragments from the polypeptide and then purified from the remaining fragments by means known to those skilled in the art. A preferred proteolytic enzyme is human or porcine elastase which cleaves plasminogen and its truncated variants between kringle regions 3-4 and 4-5 (and is thereby capable of forming peptide fragments containing kringles 1-3 and 1-4 or kringles 4 or 5 alone). For example, lys-plasminogen or glu-plasminogen may be treated with porcine or human neutrophyl elastase at a ratio of about 1:100-1:300 lys-plasminogen:elastase (preferably at a ratio of 1:150-1:250 and most preferably at a ratio of 1:150 in a buffer solution (such as Tris-HCl, NaCl, sodium phosphate and the like). Alternatively, the elastase may first be immobilized (such as to a resin) to facilitate purification of the cleavage products. The glu-plasminogen or lys-plasminogen is generally treated with human or porcine elastase at temperatures ranging from about 10°C to about 40°C and for time periods ranging from about 4 to about 24 hours depending on the extent of cleavage desired. To achieve complete digestion of glu-plasminogen, lys-plasminogen or miniplasminogen with human or porcine elastase requires exposure of these polypeptides to the enzyme for at least about 12 hours at room temperature. Varying the pH and exposure time to the enzyme results in less or partial cleavage at one or more of the susceptible cleavage sites. The cleavage products are then purified by any means well known in the art (such as column chromatography). A preferred purification scheme involves applying the cleavage products to a lysine-Sepharose column as described in Example 14.

#### Solid Phase Synthesis of Kringle 5 Peptide Fragments

The following examples will serve to further illustrate the preparation of the novel compounds of the invention:

#### EXAMPLE 1

N-Ac-Val-Leu-Leu-Pro-Asp-Val-Glu-Thr-Pro-Ser-Glu-Glu-Asp-NH<sub>2</sub>

An amide peptide synthesis column (Applied Biosystems) was placed in the peptide synthesis column position of a Perkin Elmer/Applied Biosynthesis "Synergy" peptide synthesizer, and the following synthetic sequence was used:

1. Solvating the resin with DMF for about 5 minutes;
2. Deblocking the Fmoc group from the  $\alpha$ -N-terminal of the resin-bound amino acid using 20% piperidine in DMF for about 15 minutes;
3. Washing the resin with DMF for about 5 minutes;
4. Activating the  $\alpha$ -C-terminal of amino acid No. 1 (Fmoc-Asp( $\beta$ -O<sup>t</sup>Bu), 25  $\mu$ mol) using a 0.2 M solution of HBTU (25  $\mu$ mol) and HOBT (25  $\mu$ mol) in DMSO-



NMP (N-methylpyrrolidone) and a 0.4 M solution of diisopropylethylamine (25  $\mu$ mol) in DMSO- NMP and coupling the activated amino acid to the resin;

5. Coupling the activated Fmoc-protected amino acid (prepared in step 5) to the resin-bound amino acid (prepared in step 2) in DMF for about 30 minutes;

- 5 6. Washing with DMF for 5 minutes;

7. Repeating steps 3 through 6 with the following amino acids:

<u>No.</u>	<u>Amino Acid</u>
2.	Fmoc-Glu( $\gamma$ -O <sup>t</sup> Bu)
3.	Fmoc-Glu( $\gamma$ -O <sup>t</sup> Bu)
4.	Fmoc-Ser( <sup>t</sup> Bu)
5.	Fmoc-Pro
6.	Fmoc-Thr( <sup>t</sup> Bu)
7.	Fmoc-Glu( $\gamma$ -O <sup>t</sup> Bu)
8.	Fmoc-Val
9.	Fmoc-Asp( $\beta$ -O <sup>t</sup> Bu)
10.	Fmoc-Pro
11.	Fmoc-Leu
12.	Fmoc-Leu
13.	Fmoc-Val

8. Coupling acetic acid to the  $\alpha$ -N-terminal of the resin-bound peptide *via* the conditions of steps 4 and 5.

9. Washing the resin with THF for about 5 minutes to remove DMF and shrink the resin, then drying the resin with argon for 10 minutes and nitrogen for 10 minutes more to provide clean, resin-bound peptide.

10. Cleaving of the peptide from the resin with concomitant deprotection of amino acid side chains by stirring with cleavage reagent (freshly-prepared thioanisole (100  $\mu$ L), water (50  $\mu$ L), ethanedithiol (50  $\mu$ L) and trifluoroacetic acid (1.8 mL) mixed in

the above order at -5 °C to -10 °C) at 0 °C for 10-15 minutes and then at ambient temperature for an additional 1.75 hours (plus an additional 0.5 hour for each Arg(Pmc), if present). The amount of cleavage reagent used was determined by the following formula:

<u>weight of resin with bound peptide (mg)</u>	<u>amount of cleavage reagent (<math>\mu</math>L)</u>
0-10	100
10-25	200
25-50	400
50-100	700
100-200	1200

11. Filtering and rinsing the product with neat trifluoroacetic acid, adding the filtrate in 0.5 mL portions to a centrifuge tube containing about 8 mL of cold diethyl ether, centrifuging and decanting and repeating the process until all of the peptide precipitated (if the peptide did not precipitate upon addition to ether, the mixture was extracted with aqueous 30% aqueous acetic acid (3x1mL), and the combined aqueous extracts were lyophilized to provide the product).
12. Using the peptide crude or purifying the peptide by HPLC using a 7µm Symmetry Prep C18 column (7.8 x 300 mm) with solvent mixtures varying in a gradient from 5% to 100% acetonitrile-(water, 0.1% TFA) over a period of 50 minutes followed by lyophilizing to provide 35 mg of N-Ac-Val-Leu-Leu-Pro-Asp-Val-Glu-Thr-Pro-Ser-Glu-Glu-Asp-NH<sub>2</sub>.

### EXAMPLE 2

N-Ac-Met-Phe-Gly-Asn-Gly-Lys-Gly-Tyr-Arg-Gly-Lys-Arg-Ala-Thr-Thr-Val-Thr-Gly-Thr-Pro-NH<sub>2</sub>

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Pro as amino acid No. 1. The following amino acids were added using the conditions indicated:

<u>No.</u>	<u>Amino Acid</u>
2.	Fmoc-Thr( <sup>t</sup> Bu)
3.	Fmoc-Gly
4.	Fmoc-Thr( <sup>t</sup> Bu)
5.	Fmoc-Val
6.	Fmoc-Thr( <sup>t</sup> Bu)
7.	Fmoc-Thr( <sup>t</sup> Bu)
8.	Fmoc-Ala
9.	Fmoc-Arg(Pmc)
10.	Fmoc-Lys(Boc)
11.	Fmoc-Gly
12.	Fmoc-Arg(Pmc)
13.	Fmoc-Tyr( <sup>t</sup> Bu)
14.	Fmoc-Gly
15.	Fmoc-Lys(Boc)
16.	Fmoc-Gly
17.	Fmoc-Asn(Trt)
18.	Fmoc-Gly
19.	Fmoc-Phe

20. Fmoc-Met

to provide 35 mg of N-Ac-Met-Phe-Gly-Asn-Gly-Lys-Gly-Tyr-Arg-Gly-Lys-Arg-Ala-Thr-Thr-Val-Thr-Gly-Thr-Pro-NH<sub>2</sub>.

### EXAMPLE 3

5 Ac-Gln-Asp-Trp-Ala-Ala-Gln-Glu-Pro-His-Arg-His-Ser-Ile-Phe-Thr-Pro-Glu-Thr-Asn-Pro-Arg-Ala-Gly-Leu-Glu-Lys-Asn-Tyr-NH<sub>2</sub>

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(<sup>t</sup>Bu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

<u>No.</u>	<u>Amino Acid</u>
2.	Fmoc-Asn(Trt)
3.	Fmoc-Lys(Boc)
4.	Fmoc-Glu( $\gamma$ -O <sup>t</sup> Bu)
5.	Fmoc-Leu
6.	Fmoc-Gly
7.	Fmoc-Ala
8.	Fmoc-Arg(Pmc)
9.	Fmoc-Pro
10.	Fmoc-Asn(Trt)
11.	Fmoc-Thr( <sup>t</sup> Bu)
12.	Fmoc-Glu( $\gamma$ -O <sup>t</sup> Bu)
13.	Fmoc-Pro
14.	Fmoc-Thr( <sup>t</sup> Bu)
15.	Fmoc-Phe
16.	Fmoc-Ile
17.	Fmoc-Ser( <sup>t</sup> Bu)
18.	Fmoc-His(Trt)
19.	Fmoc-Arg(Pmc)
20.	Fmoc-His(Trt)
21.	Fmoc-Pro
22.	Fmoc-Glu( $\gamma$ -O <sup>t</sup> Bu)
23.	Fmoc-Gln(Trt)
24.	Fmoc-Ala
25.	Fmoc-Ala
26.	Fmoc-Trp
27.	Fmoc-Asp( $\beta$ -O <sup>t</sup> Bu)

28. Fmoc-Gln(Trt)

to provide 40 mg of N-Ac-Gln-Asp-Trp-Ala-Ala-Gln-Glu-Pro-His-Arg-His-Ser-Ile-Phe-Thr-Pro-Glu-Thr-Asn-Pro-Arg-Ala-Gly-Leu-Glu-Lys-Asn-Tyr-NH<sub>2</sub>.

#### EXAMPLE 4

N-Ac-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH<sub>2</sub>

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Trp as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | No. | Amino Acid                            |
|-----|---------------------------------------|
| 2.  | Fmoc-Pro                              |
| 3.  | Fmoc-Gly                              |
| 4.  | Fmoc-Gly                              |
| 5.  | Fmoc-Val                              |
| 6.  | Fmoc-Asp( $\beta$ -O <sup>t</sup> Bu) |
| 7.  | Fmoc-Gly                              |
| 8.  | Fmoc-Asp( $\beta$ -O <sup>t</sup> Bu) |
| 9.  | Fmoc-Pro                              |
| 10. | Fmoc-Asn(Trt)                         |
| 11. | Fmoc-Arg(Pmt)                         |

to provide 20 mg of N-Ac-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH<sub>2</sub>.

#### EXAMPLE 5

N-Ac-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH<sub>2</sub>

The title compound was prepared using the synthetic sequence described in example 1 and using Fmoc-Tyr(<sup>t</sup>Bu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | No. | Amino Acid                            |
|-----|---------------------------------------|
| 2.  | Fmoc-Asp( $\beta$ -O <sup>t</sup> Bu) |
| 3.  | Fmoc-Tyr( <sup>t</sup> Bu)            |
| 4.  | Fmoc-Leu                              |
| 5.  | Fmoc-Lys(Boc)                         |
| 6.  | Fmoc-Arg(Pmc)                         |
| 7.  | Fmoc-Pro                              |
| 8.  | Fmoc-Asn(Trt)                         |
| 9.  | Fmoc-Thr( <sup>t</sup> Bu)            |
| 10. | Fmoc-Thr( <sup>t</sup> Bu)            |

11. Fmoc-Tyr(<sup>t</sup>Bu)  
to provide 10 mg of N-Ac-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH<sub>2</sub>.

#### EXAMPLE 6

N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH<sub>2</sub>

5 The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(<sup>t</sup>Bu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

<u>No.</u>	<u>Amino Acid</u>
2.	Fmoc-Asp(β-O <sup>t</sup> Bu)
3.	Fmoc-Tyr( <sup>t</sup> Bu)
4.	Fmoc-Leu
5.	Fmoc-Lys(Boc)
6.	Fmoc-Arg(Pmc)
7.	Fmoc-Pro

to provide N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH<sub>2</sub> (4 mg). MS (FAB) m/z 995 (M+H)<sup>+</sup>.

10

#### EXAMPLE 7

N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-NH<sub>2</sub>

The title compound was prepared using the synthetic sequence described in Example 1. The following amino acids were added using the conditions indicated:

<u>No.</u>	<u>Amino Acid</u>
2.	Fmoc-Tyr( <sup>t</sup> Bu)
3.	Fmoc-Leu
4.	Fmoc-Lys(Boc)
5.	Fmoc-Arg(Pmc)
6.	Fmoc-Leu

15 to provide N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-NH<sub>2</sub> (6 mg). MS (ESI) m/z 832 (M+H)<sup>+</sup>.

#### EXAMPLE 8

N-Ac-Pro-Glu-Lys-Arg-Tyr-Asp-Tyr-NH<sub>2</sub>

20 The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(<sup>t</sup>Bu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

<u>No.</u>	<u>Amino Acid</u>
2.	Fmoc-Asp(β-O <sup>t</sup> Bu)

3. Fmoc-Tyr(<sup>t</sup>Bu)
4. Fmoc-Arg(Pmc)
5. Fmoc-Lys(Boc)
6. Fmoc-Glu
7. Fmoc-Pro

to provide N-Ac-Pro-Glu-Lys-Arg-Tyr-Asp-Tyr-NH<sub>2</sub> (6 mg). MS (FAB) m/z (1101) (M+H)<sup>+</sup>.

#### EXAMPLE 9

##### N-Ac-Arg-Lys-Leu-Tyr-Asp-Tyr-NH<sub>2</sub>

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(<sup>t</sup>Bu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u>             |
|------------|-------------------------------|
| 2.         | Fmoc-Asp(β-O <sup>t</sup> Bu) |
| 3.         | Fmoc-Tyr( <sup>t</sup> Bu)    |
| 4.         | Fmoc-Leu                      |
| 5.         | Fmoc-Lys(Boc)                 |
| 6.         | Fmoc-Arg(Pmc)                 |

to provide N-Ac-Arg-Lys-Leu-Tyr-Asp-Tyr-NH<sub>2</sub> (8 mg). MS (ESI) m/z 898 (M+H)<sup>+</sup>.

#### EXAMPLE 10

##### N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH<sub>2</sub> (SEQ ID NO: 13)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(<sup>t</sup>Bu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u>              |
|------------|--------------------------------|
| 2.         | Fmoc-Asp(β-O <sup>t</sup> Bu)  |
| 3.         | Fmoc-3-I-Tyr( <sup>t</sup> Bu) |
| 4.         | Fmoc-Leu                       |
| 5.         | Fmoc-Lys(Boc)                  |
| 6.         | Fmoc-Arg(Pmc)                  |
| 7.         | Fmoc-Pro                       |

to provide N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH<sub>2</sub> (2 mg). MS (ESI) m/z (1121) (M+H)<sup>+</sup>.

#### EXAMPLE 11

N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH<sub>2</sub> (SEQ ID NO: 14)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-3-I-Tyr(<sup>t</sup>Bu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u>                     |
|------------|---------------------------------------|
| 2.         | Fmoc-Asp( $\beta$ -O <sup>t</sup> Bu) |
| 3.         | Fmoc-Tyr( <sup>t</sup> Bu)            |
| 4.         | Fmoc-Leu                              |
| 5.         | Fmoc-Lys(Boc)                         |
| 6.         | Fmoc-Arg(Pmc)                         |
| 7.         | Fmoc-Pro                              |

5 to provide N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH<sub>2</sub> (2.5 mg). MS (ESI) m/z 1121 (M+H)<sup>+</sup>.

#### EXAMPLE 12

N-Ac-Lys-Leu-Tyr-Asp-NH<sub>2</sub>

10 The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Asp( $\beta$ -O<sup>t</sup>Bu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u>          |
|------------|----------------------------|
| 2.         | Fmoc-Tyr( <sup>t</sup> Bu) |
| 3.         | Fmoc-Leu                   |
| 4.         | Fmoc-Lys                   |

to provide 2 mg of N-Ac-Lys-Leu-Tyr-Asp-NH<sub>2</sub> (2 mg).

15

#### EXAMPLE 13

Preparation and separation of a mixture N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I<sup>125</sup>-Tyr<sup>535</sup>-NH<sub>2</sub> and N-Ac-Pro-Arg-Lys-Leu-3-I<sup>125</sup>-Tyr<sup>533</sup>-Asp-Tyr-NH<sub>2</sub> (SEQ ID NO: 13) and (SEQ NO: ID 14), respectively.

20 To a solution of 30  $\mu$ g of N-acetyl-prolyl-arginyl-lysyl-leucyl-tyrosyl-aspartyl-tyrosylamide in 80 mL of phosphate buffered saline (PBS) was added one iodobead (Pierce, Rockford, IL) and 100  $\mu$ Ci of NaI<sup>125</sup>. After 10 minutes, the excess NaI<sup>125</sup> reagent was removed by applying the reaction mixture to a Waters C18-Light SepPack column and eluting with water then 0.1% TFA in 1:1 CH<sub>3</sub>CN/water and collecting 3 X 200 $\mu$ L fractions  
25 to provide a mixture of Tyr<sup>533</sup>- and Tyr<sup>535</sup>- radiolabeled peptides.

The hot peptide mixture was coinjected onto a C18 HPLC column with an equimolar solution of cold carriers N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH<sub>2</sub> and N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH<sub>2</sub>, the elution times of which had been predetermined as 36 and 38 minutes, respectively. Repeated elutions with the solvent system in Example 1 and lyophilization of the combined, relevant fractions provided the desired compound N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH<sub>2</sub> with a minimal impurity N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH<sub>2</sub>.

### General Methodologies

#### EXAMPLE 14

##### Isolation and Purification of Kringle 5 Peptide Fragments

The kringle 5 peptide fragments were prepared from the digestion of Lys plasminogen (Lys-HPg, Abbott Laboratories, Abbott Park, IL) with porcine elastase (SIGMA, St. Louis, MO) by a modification of the method of Powell *et al.* (Arch Biochem. Biophys. 248(1): 390-400 (1986), which is hereby incorporated herein by reference). 1.5 mg of porcine elastase was incubated with 200 mg of Lys-HPg in 50 mM Tris-HCl pH 8.0 and rocked overnight at room temperature. The reaction was terminated by the addition of DPF (diisopropyl fluorophosphate, SIGMA) to a final concentration of 1 mM. The mixture was rocked for an additional 30 minutes, dialysed against 50 mM Tris pH 8.0 overnight and concentrated. The cleaved plasminogen was placed over a 2.5 cm X 15 cm lysine-Sepharose 4B column (Brockway, W.J. and Castellino, F.J., Arch. Biochem. Biophys. 151: 194-199 (1972), which is hereby incorporated by reference) and equilibrated with 50 mM Tris pH 8.0 until an absorbance of 0.05 (at 280 nm) was reached. (This step was performed to remove any fragments containing a kringle 1 region and/or a kringle 4 region (both of which bind lysine)). The non-absorbed kringle 5 peptide fragments were dialysed against 50 mM Na<sub>2</sub>PO<sub>4</sub> buffer, pH 5.0 then applied to a BioRad Mono-S column equilibrated with the same buffer. The cleaved kringle 5 portion, uncut mini-HPg and remaining protease domain fraction were eluted with a 0-20%, 20-50% and 50-70% step gradient of 20 mM Phosphate/1 M KCl pH 5.0. The kringle 5 peptide fragments eluted at the 50% step as determined by gel electrophoresis. The collected peak was dialysed overnight against 20 mM Tris pH 8.0.

The separated kringle 5 fragments were determined to be at least 95% pure by FPLC chromatography and DodSO<sub>4</sub>/PAGE with silver staining (Coomassie Blue). Sequence analysis of the amino terminal portion of the purified fragments revealed the presence of three polypeptides having  $\alpha$ -N-terminus sequences of VLLPDVETPS, VAPPPVVLL and VETPSEED which correspond to amino acid positions Val<sup>449</sup>-Ser<sup>458</sup>, Val<sup>443</sup>-Leu<sup>450</sup> and Val<sup>454</sup>-Asp<sup>461</sup> of SEQ ID. NO: 1, respectively.



### EXAMPLE 15

#### Endothelial Proliferation Assay

The *in vitro* proliferation of endothelial cells was determined as described by Lingen, *et al.* in *Laboratory Investigation*, **74**: 476-483 (1996), which is hereby incorporated herein by reference, using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega Corporation, Madison, WI). Bovine capillary (adrenal) endothelial cells were plated at a density of 1000 cells per well in a 96-well plate in Dulbecco's Modified Eagle Medium (DMEM) containing 10% donor calf serum and 1% BSA (bovine serum albumin, GIBCO BRL, Gaithersburg, MD). After 8 hours, the cells were starved overnight in DMEM containing 0.1% BSA then re-fed with media containing specified concentrations of inhibitor and 5 ng/mL bFGF (basic fibroblast growth factor). The results of the assay were corrected both for unstimulated cells (i.e. no bFGF added) as the baseline and for cells stimulated with bFGF alone (i.e. no inhibitor added) as the maximal proliferation. When multiple experiments were combined, the results were represented as the percent change in cell number as compared to bFGF alone.

### EXAMPLE 16

#### Endothelial Cell Migration Assay

The endothelial cell migration assay was performed essentially as described by Polverini, P.J. *et al.*, *Methods Enzymol*, **198**: 440-450 (1991), which is hereby incorporated herein by reference. Briefly, bovine capillary (adrenal) endothelial cells (BCE, supplied by Judah Folkman, Harvard University Medical School) were starved overnight in DMEM containing 0.1% bovine serum albumin (BSA). Cells were then harvested with trypsin and resuspended in DMEM with 0.1% BSA at a concentration of  $1.5 \times 10^6$  cells/mL. Cells were added to the bottom of a 48-well modified Boyden chamber (Nucleopore Corporation, Cabin John, MD). The chamber was assembled and inverted, and cells were allowed to attach for 2 hours at 37°C to polycarbonate chemotaxis membranes (5  $\mu$ m pore size) that had been soaked in 0.1% gelatin overnight and dried. The chamber was then reinverted and test substances were added to the wells of the upper chamber (to a total volume of 50  $\mu$ L); the apparatus was then incubated for 4 hours at 37°C. Membranes were recovered, fixed and stained (DiffQuick, Fisher Scientific, Pittsburgh, PA) and the number of cells that had migrated to the upper chamber per 10 high power fields were counted. Background migration to DMEM + 0.1% BSA was subtracted and the data reported as the number of cells migrated per 10 high power fields (400X) or when results from multiple experiments were combined, as the percent inhibition of migration compared to a positive control. The results are shown in Table 1.

### EXAMPLE 17

#### Effect of Kringle 5 Peptide Fragments on Endothelial Cell Proliferation *in vitro*

The effect of kringle 5 peptide fragments on endothelial cell proliferation was determined *in vitro* using the above described endothelial cell proliferation assay. For these experiments, kringle 5 peptide fragments was prepared as illustrated in Examples 1 through 14 and tested at various concentrations ranging from about 100 to 1000 pM with bFGF used as a maximum proliferation control. The kringle 5 peptide fragment SEQ ID NO: 3 was effective at inhibiting BCE cell proliferation in a dose-dependent manner. The concentration of kringle 5 peptide fragment SEQ ID NO: 3 required to reach 50% inhibition (ED<sub>50</sub>) was determined at about 300 pM. In contrast, the ED<sub>50</sub> of kringles 1-4 was shown to be 135 nM.

A summary of the effect of other kringle peptide fragments on inhibition of BCE cell proliferation is shown in Table 1. The kringle 3 peptide fragment was least effective at inhibiting BCE cell proliferation (ED<sub>50</sub> = 460 nM), followed by the kringle 1 peptide fragment (ED<sub>50</sub> = 320 nM), kringle 1-4 peptide fragments (ED<sub>50</sub> = 135 nM) and kringles 1-3 peptide fragments (ED<sub>50</sub> = 75 nM). The kringle 5 peptide fragment was the most effective at inhibiting BCE cell proliferation with an ED<sub>50</sub> of 0.3 nM.

### EXAMPLE 18

#### Effect of Kringle 5 Peptide Fragments on Endothelial Cell Migration *in vitro*

The effect of kringle 5 peptide fragments on endothelial cell migration was also determined *in vitro* using the above described endothelial cell migration assay. Kringle 5 peptide fragments inhibited BCE cell migration in a dose-dependent fashion with an ED<sub>50</sub> of approximately 300 pM. At the concentration of kringle 5 peptide fragments required for maximal inhibition of BCE cells, PC-3 cells and MDA 486 cells were also inhibited. This result, taken together with the result in Example 2, indicates that the inhibition of stimulated proliferation and migration of BCE cells by kringle 5 peptide fragments is both potent and specific to endothelial cells and not to normal or tumor cells.

The foregoing are merely illustrative of the invention and are not intended to limit the invention to the disclosed compounds. Variations and changes which are obvious to one skilled in the art are intended to be within the scope and nature of the invention which are defined in the appended claims.

Table 1 shows a summary of ED<sub>50</sub> values obtained from the inhibition of various kringle fragments on BCE cell proliferation and cell migration *in vitro*. In the table, kringle peptide fragments are labeled according to their corresponding sequence homology to SEQ ID NO: 1. The symbol "\*" indicates data taken from Marti, D., *et al.*, Eur. J. Biochem.,

219:455-462 (1994), which is hereby incorporated by reference and the symbol “-” indicates no data.

Table 1

Protein Fragment from SEQ ID NO: 1	Antiproliferative Activity of BCE Cells (ED <sub>50</sub> )	Migratory Inhibition of HMVEC Cells (ED <sub>50</sub> )
kringles 1-4 (angiostatin)*	135 nM	160 nM
kringle 1 (Tyr <sup>80</sup> -Glu <sup>163</sup> )*	320 nM	-
kringle 2 (Glu <sup>161</sup> -Thr <sup>245</sup> )*	no activity	-
kringle 3 (Thr <sup>253</sup> -Ser <sup>335</sup> )*	460 nM	-
kringle 4 (Val <sup>354</sup> -Val <sup>443</sup> )*	no activity	-
kringles 1-3 (Tyr <sup>80</sup> -Pro <sup>353</sup> )*	75 nM	60 nM
kringles 2-3 (Glu <sup>161</sup> -Ser <sup>335</sup> )*	-	-
kringle 5 (Val <sup>443</sup> -Ala <sup>543</sup> )	250 pM	200 pM
kringle 5 (Val <sup>449</sup> -Ala <sup>543</sup> )	-	240 pM
kringle 5 (Val <sup>454</sup> -Ala <sup>543</sup> )	-	220 pM
kringle 5 (Val <sup>443</sup> -Phe <sup>546</sup> )	60 nM	55 nM
kringle 5 (Val <sup>449</sup> -Phe <sup>546</sup> )	-	-
kringle 5 (Val <sup>454</sup> -Phe <sup>546</sup> )	-	-
kringles 4-5 (Val <sup>355</sup> -Ala <sup>543</sup> )	-	280 pM
kringles 4-5 (Val <sup>355</sup> -Phe <sup>546</sup> )	-	-
N-Ac-Val <sup>449</sup> -Asp <sup>461</sup> -NH <sub>2</sub>	-	> 1 mM
N-Ac-Met <sup>463</sup> -Pro <sup>482</sup> -NH <sub>2</sub>	-	> 1 mM
N-Ac-Gln <sup>484</sup> -Tyr <sup>511</sup> -NH <sub>2</sub>	-	>100 μM
N-Ac-Arg <sup>513</sup> -Trp <sup>523</sup> -NH <sub>2</sub>	-	500 pM
N-Ac-Tyr <sup>525</sup> -Tyr <sup>535</sup> -NH <sub>2</sub>	-	200 pM
N-Ac-Pro <sup>529</sup> -Tyr <sup>535</sup> -NH <sub>2</sub>	-	120 pM
N-Ac-Pro <sup>529</sup> -Asp <sup>534</sup> -NH <sub>2</sub>	-	123 pM
N-Ac-Pro <sup>150</sup> -Tyr <sup>156</sup> -NH <sub>2</sub>	-	160 nM
N-Ac-Arg <sup>530</sup> -Tyr <sup>535</sup> -NH <sub>2</sub>	-	80 pM
N-Ac-Pro-Arg-Lys-Leu-3-I- Tyr-Asp-Tyr-NH <sub>2</sub>	-	> 100 nM
N-Ac-Pro-Arg-Lys-Leu-Tyr- Asp-3-I-Tyr-NH <sub>2</sub>	-	400 pM
N-Ac-Lys <sup>531</sup> -Tyr <sup>534</sup> -NH <sub>2</sub>	-	-

**EXAMPLE 19****Recombinant Expression of Kringle 5 Fragments in *Pichia pastoris***

A. **Production of cDNAs Encoding Kringle 5 Fragments by PCR:** PCR was employed to generate cDNA fragments which encode kringle 5 peptide fragments having amino acid sequences from (1) amino acid positions 450-543 of SEQ ID NO:1 (hereinafter, K5A), (2) amino acid positions 450-546 of SEQ ID NO:1 (hereinafter K5F), (3) amino acid positions 355-543 of SEQ ID NO:1 (hereinafter K4-5A), and (4) amino acid positions 355-546 of SEQ ID NO:1 (hereinafter K4-5F) for cloning and expression in both eukaryotic and prokaryotic hosts. DNA fragments were generated using a cDNA encoding human plasminogen (obtained from Dr. E. Reich, State University of New York, Stony Brook, NY) as template and sets of forward and reverse primers (obtained from Operon Technologies, Inc. Alameda, CA) shown below:

5' - ATTAATGGATCCTTGGACAAGAGGCTGCTTCCAGATGTAGAGACT - 3'	SEQ ID NO:2
5' - ATTAATGGATCCTTGGACAAGAGGGTCCAGGACTGCTACCATGGT - 3'	SEQ ID NO:3
5' - ATTAATCTCGAGGCATGCTTAGGCCGCGACACTGATGGACA - 3'	SEQ ID NO:4
5' - ATTAATCTCGAGGCATGCTTAAATGAAGGGGCGCGACACT - 3'	SEQ ID NO:5

PCR amplifications were performed using primer sets SEQ ID NO:2 and SEQ ID NO:4 (for K5A), SEQ ID NO:2 and SEQ ID NO:5 (for K5F), SEQ ID NO:3 and SEQ ID NO:8 (for K4-5A) and SEQ ID NO:3 and SEQ ID NO:5 (for K4-5A) under standard PCR conditions, i.e. in a total reaction volume of 100  $\mu$ L containing 200  $\mu$ M of each dNTP wherein N was A, T, G and C, 0.2  $\mu$ M of each primer, approximately 10 ng of template DNA and 1 unit of Vent® DNA polymerase (New England Biolabs). Amplifications were carried out for a total of 25 cycles (1 cycle = 94°C for one minute, 48°C for two minutes, 72°C for 1 minute) on a DNA Thermal Cycler 480 (Perkin Elmer, Foster City, CA). After amplification, PCR products were gel purified, digested with *Bam*HI and *Xho*I (New England Biolabs), ligated to a modified *Pichia* expression vector (pHil-D8, see below) cut with the same enzymes and transformed into HB101 cells (BioRad) by electroporation. DNA was prepared from individual clones and subjected to restriction enzyme digestion and sequence analysis to identify clones that contained inserts with the correct sequence and in the proper orientation. Plasmids from positively identified clones were then transformed into *Pichia pastoris* strain GS115 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's directions. To identify positive clones in *Pichia*, cells were grown in 5 mL of BMGY medium (Invitrogen) at 29°C overnight, collected by centrifugation and resuspended in 0.5 mL BMMY medium (Invitrogen) for expression. After incubation at

29°C for two days, culture supernatants were collected and aliquots subjected to SDS-PAGE and western blot analysis according to known techniques. An SDS-PAGE gel is shown in FIG. 6.

B. Construction of Expression Vector pHil-D8: The *Pichia* expression vector, pHil-D8, was constructed by modification of vector pHil-D2 (Invitrogen) to include a synthetic leader sequence for secretion of a recombinant protein (see FIG. 5). The leader sequence, 5'-ATGTTCTCTCCAATTTTGTCCTTGGAAATTATTTTAGCTTTGGCTACTTTGCAATCTGTCTTCGCTCAGCCAGTTATCTGCACTACCGTTGGTTCCGCTGCCGAGGGATCC-3' (SEQ ID NO:9) encodes a PHO1 secretion signal (single underline) operatively linked to a pro-peptide sequence (bold highlight) for KEX2 cleavage. To construct pHil-D8, PCR was performed using pHil-S1 (Invitrogen) as template since this vector contains the sequence encoding PHO1, a forward primer (SEQ ID NO:7) corresponding to nucleotides 509-530 of pHil-S1 and a reverse primer (SEQ ID NO:8) having a nucleotide sequence which encodes the latter portion of the PHO1 secretion signal (nucleotides 45-66 of SEQ ID NO:9) and the pro-peptide sequence (nucleotides 67-108 of SEQ ID NO:9). The primer sequences (obtained from Operon Technologies, Inc. Alameda, CA) were as follows:

5' -GAAACTTCCAAAAGTCGCCATA-3'	SEQ ID NO:7
5' -ATTAATGAATTCCTCGAGCGGTCCGGGATCCCTCGGCAGCGGAACCAA CGGTAGTGCAGATAACTGGCTGAGCGAAGACAGATTGCAAAGTA-3'	SEQ ID NO:8

Amplification was performed for 25 cycles as described in Example 19. The PCR product (approximately 500 bp) was gel-purified, cut with *Bln*I and *Eco*RI and ligated to pHil-D2 cut with the same enzymes. The DNA was transformed into *E. coli* HB101 cells and positive clones identified by restriction enzyme digestion and sequence analysis. One clone having the proper sequence was designated as pHil-D8.

## EXAMPLE 20

### Recombinant Expression of Kringle 5 Peptide Fragments in Bacteria

Restriction or other modifying enzymes as well as other reagents used were obtained from commercial sources. Primers were synthesized at Abbott Laboratories on an automatic synthesizer by standard methods known in the art.

DNAs of kringle 5 peptide fragments were also generated by PCR amplification for cloning and expression in bacterial cells (*E. coli*). The general approach taken was to generate PCR fragments of desired coding regions, with and without termination codons, kinase the ends, and clone the fragments directly into vectors of choice. Vector constructs were then transformed into appropriate host cells and colonies screened by PCR with vector

primers to confirm the presence of an insert. To determine the orientation of an insert, PCR reactions showing insert positive clones were subjected to directional PCR using 1 vector primer and 1 insert primer.

A. Preparation of blunt-end, phosphatased vectors: A description of expression vectors useful for bacterial production of kringle 5 peptide fragments is shown in Table 2.

Table 2

Vector	Source	Restriction Enzymes	Fusion
UpET	Abbott-modified pET21d	<i>SapI</i>	None
UpET-HTH	Abbott-modified pET21d	<i>SapI</i>	N-Terminal His6-Thrombin recognition
UpET-Ubi	Abbott-modified pET21d	<i>SapI</i>	N-Terminal His6-Ubiquitin-Enterokinase recognition
pET32a	Novagen	<i>NcoI</i> + <i>XhoI</i>	Thioredoxin, Enterokinase recognition
pGEX-4T-2	Pharmacia®	<i>EcoRI</i> + <i>NotI</i>	GST
pCYB3	New England Biolabs	<i>NcoI</i> + <i>SapI</i>	C-terminal intein

All vectors were first isolated and purified using Qiagen columns in accordance with the manufacturer's instructions (QIAGEN, Inc., Santa Clarita, CA). Vector DNA (1 µg) was digested with appropriate restriction enzymes (see Table 2) in 20 µL of NEB4 buffer (New England Biolabs) containing 100 µg/mL bovine serum albumin (BSA). The reaction was centrifuged briefly, 20 µL of deionized H<sub>2</sub>O, 0.4 µL of dNTP mix (Pharmacia®; 20 mM each dNTP) and 0.25 µL of cloned *pfu* DNA polymerase (Stratagene®; 2.5 units/µL) was added and the reaction mixture incubated at 65°C for 20 minutes to fill in the vector ends. The reaction mixture was again centrifuged briefly and 4 µL of diluted calf intestinal phosphatase (GIBCO BRL, Gaithersburg, MD; 5 units total) was added. The mixture was then incubated at 50°C for one hour. Five 5 µL of 10%SDS, 2 µL of 5 M NaCl, 2 µL of 0.5 M EDTA and 45 µL of H<sub>2</sub>O were added, the reaction was centrifuged briefly and then incubated at 65°C for 20 minutes. The reaction was then extracted three times with buffer-saturated phenol-chloroform (GIBCO BRL) and once with chloroform. The aqueous phase was purified through a CHROMA SPIN™ 1000 TE column (CLONTECH, Palo Alto, CA).

B. Generation of DNA Fragments by PCR: PCR primers were designed and ordered based upon the published sequence for human plasminogen (see SEQ ID NO:12) and are shown below:

5' -GTCCAGGACTGCTACCAT-3'	SEQ ID NO:10
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5' -CTGCTTCCAGATGTAGAGA-3'	SEQ ID NO:11
5' -TTATTAGGCCGCACACTGAGGGA-3'	SEQ ID NO:13

Unless otherwise noted, all PCRs were performed with *pfu* DNA polymerase and buffer (Stratagene®), using 200 µM each dNTP and 1 µM each primer. Primer sets used were SEQ ID NO:11 and SEQ ID NO:13 (for K5A), and SEQ ID NO:10 and SEQ ID NO:13 (for K4-5A). Vector pHil-D8 containing the K4-K5A (described in Example 19) was used as template. Prior to use as a template, this DNA was digested with *DraI* (which makes multiple cuts outside of the kringle regions) in order to eliminate background due to the pHil-D8 vector in transformations. Approximately 10 ng of template was used per 50 µL PCR reaction. PCR reactions were run at 94°C for 2 mins.; then for 15 cycles of 94°C, 30 sec.; 49°C, 1 min.; 72°C, 4 mins; and 72°C, 7 mins. After the PCR reaction, 0.5 µL of 100 mM ATP and 5 units of T4 Kinase was added and the reaction incubated at 37°C for 20 mins. to kinase the ends. The reaction was then heated at 68°C for 15 mins. and purified over an S400-HR spin column (Pharmacia®) for use in ligations.

C. Ligation of PCR Fragments into Expression Vectors: Six recombinant constructions (specifically, (i) K5A in UpET-PS3, (ii) K5A in pET32a, (iii) K4-5A in UpET-PS3, (iv) K4-5A in UpET-Ubi, (v) K4-5A in pET32a and (vi) K4-5A in pGEX-4T-2) were made as follows: blunt-end, phosphatased vector (1 µL from step A above) and PCR fragment (1 µL from step B above) were ligated in a total volume of 5.5 µL using a Rapid Ligation Kit as per the manufacturers instructions (BOEHRINGER-MANNHEIM Corp., Indianapolis, IN). Ligation mixture (1 µL) was then transformed into 20 µL of competent cells (XL1-Blue Supercompetent cells or XL2-Blue Ultracompetent cells (Stratagene®)) as per the manufacturer's instructions. Recombinant cells were selected on LB-Amp agar plates (MicroDiagnostics, Lombard, IL).

D. Expression Studies: pGEX vectors were expressed in *E. coli* XL1-Blue or XL2-Blue. All other vectors were isolated and retransformed into *E. coli* BL21(DE3) (Novagen) as per the manufacturer's instructions. Individual colonies were inoculated into 2.5 mL of LB/Amp and shaken at 225 rpm, 37°C, overnight. Overnight culture (0.5 mL) was then inoculated into 50 mL of LB/Amp in a 250 mL flask and shaken at 225 rpm, 37°C to an OD600 of 0.5 - 0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG, 100 mM) was then added to a final concentration of 1 mM. The culture was shaken at 225 rpm, 30°C for 3 hours before being spun down. Samples were prepared for SDS-PAGE in accordance with known techniques. Preliminary experiments showed that cells having K5A/pET32a, K4-5A/pET32a and K4-5A/pGEX produced the most recombinant protein. Cultures of these clones was then analyzed for soluble vs. insoluble expression by SDS-PAGE. As FIG. 7 shows, K5A/pET32a produced recombinant protein that is almost completely soluble

(compare lanes S and P of Trx-K5A), whereas K4-5A/pET32a and K4-5A/pGEX produced about 75% soluble protein .

E. Construction of Abbott-modified vectors

i. VB1, VB2, VB3 and VB4 Cassette Preparation: VB1, VB2, VB3, and VB4 were made as synthetic DNAs using techniques well known to those of ordinary skill in the art. The sequences of synVB1, synVB2, synVB3, and synVB4 are shown below:

synVB1	5' -AGCGTCTCATGAAGAGCTGGCTCACCTTCGGGTGGGCCTTTCTGC GCCTTGGCGCGCCAACCTTAATTAACCGGGAGCCCGCCTAATGAGCGG GCTTTTTTTTGCTCTTCATAGTGAAGTGAAGACGTCG-3'	SEQ ID NO:14
synVB2	5' -AGCGTCTCAGGTGGTGGTCATCACCATCACCATCACGGTGGTGGT CTGGTGCCGCGCGGCAGCTGAAGAGCTGGCTCACCTTCGGGTGGGCCT TTCTGCGCCTTGGCGCGCCAACCTTAATTAACCGGGAGCCCGCCTAAT GAGCGGGCTTTTTTTTGCTCTTCACGAGACGTCG-3'	SEQ ID NO:15
synVB3	5' -AGCGTCTCAGGTGGTGGTCATCACCATCACCATCACGGTGGTGGT TGAAGAGCTGGCTCACCTTCGGGTGGGCCTTTCTGCGCCTTGGCGCGC CAACCTTAATTAACCGGGAGCCCGCCTAATGAGCGGGCTTTTTTTTG TCTTCACGAGACGTC-3'	SEQ ID NO:16
synVB4	5' -AGCGTCTCAGGTGGTGGTCATCACCATCACCATCACGGTGGTGGT GATGACGATGACAAGTGAAGAGCTGGCTCACCTTCGGGTGGGCCTTTC TGCGCCTTGGCGCGCCAACCTTAATTAACCGGGAGCCCGCCTAATGAG CGGGCTTTTTTTTGCTCTTCACGAGACGTCG-3'	SEQ ID NO:17

Each synthetic sequence was made double stranded and cloned into pCR-Script Cam™ (Stratagene®) as per the manufacturer's instructions; clones with the correct sequence were then isolated by standard procedures. Five µg of purified DNA was digested with 8 units of *Bsm*BI at 55°C in 20 µL reactions in 1X NEB4 Buffer containing 100 µg/mL BSA. The reaction was centrifuged briefly, 20 µL of deionized H<sub>2</sub>O, 0.4 µL of dNTP mix (Pharmacia®; 20 mM each dNTP) and 0.25 µL cloned *pfu* DNA polymerase (Stratagene®; 2.5 units per µl) were added and the reaction was incubated at 65°C for 20 minutes to fill in the ends. The DNA was then run on a 3% MetaPhor™ Agarose gels (FMC, Rockland, Maine) in 0.5X Tris-Acetate-EDTA buffer (TAE). The cassette band was cut out and the DNA was eluted by freezing the gel and centrifuging the buffer through an Ultrafree™ Probind cartridge (MILLIPORE Corp., Bedford, MA), followed by isopropanol precipitation using Pellet Paint™ (Novagen) as a carrier. The DNA (cfVB1, cfVB2, cfVB3, and cfVB4) was rinsed with 70% ethanol, dried briefly and resuspended in 25µL of Tris-EDTA (TE) buffer.



ii. Construction of UpET: Vector pET21d (Novagen) was digested with *SapI*, treated first with T4 DNA Polymerase + dGTP, then Mung Bean Nuclease, then DNA polymerase I Klenow fragment and religated. Individual colonies were screened to select a plasmid in which the existing *SapI* site had been eliminated. This DNA was then digested with *NcoI* + *BamHI* and ligated to 5'-CATGTGAAGAGC-3' (SEQ ID NO:19) + 5'-GATCGCTCTTCA-3' (SEQ ID NO:20) to introduce a single *SapI* site. Purified, verified cloned DNA was cut with *SapI* + *HindIII*, blunted and phosphatased as described above, ligated with the cfVB1 cassette, transformed into *E. coli* and plated on LB-Amp plates. Colonies were picked with sterile pipette tips onto LB-Amp agar plates and into 20  $\mu$ L of AmpliTaq® PCR mix (Perkin Elmer) in Costar Thermowell plates containing 1  $\mu$ M of each vector primers 5'-AGATCTCGATCCCGCGAA-3' (forward primer, SEQ ID NO:21) and 5'-ATCCGGATATAGTTCCTC-3' (SEQ ID NO:22). Reactions were heated to 94° for 5', then cycled using a GeneAmp 9600 thermal cycler for 30 cycles of 94°, 30 seconds; 40°, 1 minute; 72°, 2 minutes. 10  $\mu$ L of each reaction was run on agarose gels. To determine the orientation of the cassette, 0.25  $\mu$ L of a PCR screen with the correct size was added to a fresh reaction containing the reverse vector primer and a cassette primer 5'-CGGGCTTTTTTTTGCTCTTCA-3' (SEQ ID NO:23). Reactions were cycled as above for an additional 10 cycles. Final vectors were sequenced using standard procedure and one clone designated as UpET.

iii. Construction of UpET-HTH: UpET was digested with *SapI* and prepared for blunt, phosphatased cloning. It was ligated to the cfVB2 cassette, transformed, colonies screened and sequenced as for the cfVB1 ligation above.

iv. Construction of UpET-H: UpET was digested with *SapI* and prepared for blunt, phosphatased cloning. It was ligated to the cfVB3 cassette, transformed, colonies screened and sequenced as for the cfVB1 ligation above.

v. Construction of UpET-Ubi: A PCR fragment for *S. cerevisiae* ubiquitin was generated using Ultra DNA polymerase and buffer (Perkin Elmer), 40  $\mu$ M each dNTP, 1  $\mu$ M each of the primers 5'-CAGATTTTCGTCAAGACTT-3' (Ubi-5p, SEQ ID NO:24) and 5'-ACCACCTCTTAGCCTTAG-3' (Ubi-3p, SEQ ID NO:25) and 1.75  $\mu$ g of yeast DNA at 94°C, 2 mins. then 25 cycles of 94°C, 1 min.; 40°C, 1 min.; 72°C, 2 mins.; then 72°C for 7 mins.. A PCR fragment was generated from 20 ng of pET15b (Novagen) using the primers 5'-CATGGTATATCTCCTTCTT-3' (pET3p-ATG, SEQ ID NO:26) and 5'-TGAGCAATAACTAGCATAAC-3' (T7RevTerm, SEQ ID NO:27) at 94°C, 2 mins. then 10 cycles of 94°C, 45 sec.; 42°C, 1 min.; 72°C, 15 mins.; then 72°C for 7 mins.. The Ubiquitin and pET15b-derived PCR fragments were gel-purified and ligated together using BRL T4 ligase and ligase buffer. A T7 promoter-ubiquitin (T7-ubiquitin) PCR fragment was then generated using the ligation as template and Ultra DNA polymerase and the

primers 5'-AGATCTCGATCCCGCGAA-3' (pET5p, SEQ ID NO:28) and SEQ ID NO:25 at 94°C, 2 mins. then 25 cycles of 94°C, 30 sec.; 42°C, 1 min.; 72°C, 3 mins.; then 72°C, 7 mins.. The T7-ubiquitin PCR fragment was gel purified.

A PCR fragment for mature human Stromelysin was generated using Ultra DNA polymerase (as above) with the primer 5'-TTAGGTCTCAGGGGAGT-3' (Strom-3p, SEQ ID NO:29) and kinased primer 5'-TTCAGAACCTTTCCTGGCA-3' (Strom-5p, SEQ ID NO:30) and approximately 20 ng of template (i.e. stromelysin cloned into pET3b (Novagen)) at 94°C, 2' then 15 cycles of 94°C, 1 min.; 44°C, 1 min.; 72°C, 2 mins., then 72°C for 7 mins.. The stromelysin PCR reaction (10µL) was ligated with 100 pMol of annealed oligos 5'-AGCGGCGACGACGACGACAAG-3' (Ek-Cut-5p, SEQ ID NO:31) and 5'-CTTGTCGTCGTCGTCGCCGCT-3' (Ek-Cut-3p, SEQ ID NO:32 coding for an Enterokinase cleavage site) in 40 µL of BRL ligase and ligase buffer. An enterokinase site - mature stromelysin (Ek-Stromelysin) PCR fragment was generated using 1 µL of this ligation as a template, primers SEQ ID NO:29 and kinased SEQ ID NO:31, Ultra DNA polymerase and buffer at 94°C, 2 mins.; then 10 cycles of 94°C, 1 min.; 44°C, 1 min.; 72°C, 2 mins., then 72° for 7 mins.. The Ek-Stromelysin PCR fragment was gel purified.

The T7-ubiquitin and Ek-stromelysin PCR fragments were ligated together in BRL ligase and ligase buffer. A T7-ubiquitin-Ek-stromelysin PCR fragment was then generated using the ligation as template and Ultra DNA polymerase and the primers SEQ ID NO:28 and SEQ ID NO:29 at 94°C, 2' then 25 cycles of 94°C, 30 sec.; 42°C, 1 min.; 72°C, 6 mins., then 72°C for 7 mins.

A PCR fragment was generated using the stromelysin-pET3b plasmid template with the primers SEQ ID NO:26 and SEQ ID NO:30 with KlenTaq (AB Peptides, St. Louis, MO) and *pfu* DNA polymerases at 94°C, 2' then 15 cycles of 94°C, 30 sec.; 42°C, 2 mins.; 68°C, 20 mins.. This PCR fragment was mixed with the T7-Ubiquitin-Ek-Stromelysin PCR fragment and transformed into BRL DH5α maximum efficiency competent cells. Correct clones were identified by isolation of plasmid DNA, transfection into BL21(DE3), and expression studied as described above.

A PCR fragment for Ubiquitin-Ek was generated from a correct T7-Ubiquitin-Ek-Stromelysin expression plasmid with the primers SEQ ID NO:24 and SEQ ID NO:32 and *pfu* DNA polymerase at 94°C, 2' then 20 cycles of 94°C, 30 sec.; 40°C, 1 min.; 72°C, 3 mins., 72°C, 7 mins.. The fragment was purified over a Pharmacia S-400 HR Spin column and ligated to the VBC1 cassette using the Rapid DNA Ligation kit. A PCR fragment was generated using the ligation as template and the primers SEQ ID NO:24 and 5'-TGAAGAGCAAAAAAGCCCG-3' (SEQ ID NO:33) and *pfu* DNA polymerase at 94°C, 2 mins. then 20 cycles of 94°C, 30 sec.; 40°C, 1 min.; 72°C, 2 mins., 72°C, 7 mins.. The PCR fragment was kinased and ligated to Upet-H prepared for blunt, phosphatased cloning.

The ligation was transformed into competent cells and colonies were screened by PCR as above. Plasmid DNA was sequenced to identify correct clones of UpET-Ubi.

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## Example 21

### Preparation of Kringles 1-5 Peptide Fragments

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A. Method 1: Kringle fragment 1-5 (K1-5) was prepared from the limited digestion of Lys-HPg (Abbott Labs) with porcine elastase (Sigma Chemical Co., St. Louis, MO) by the method of Powell and Castellino, 1986, *supra*. Briefly, 1.5 mg of elastase was incubated with 200 mg of Lys-HPg in 50 mM Tris-HCl pH 8.0 and rocked for four hours at room temperature. The reaction was terminated by the addition of diisopropyl fluorophosphate (DFP, Sigma Chemical Co., St. Louis, MO) to a final concentration of 1 mM. The mixture was rocked for an additional 30 minutes, dialyzed against 50 mM Tris pH 8.0 overnight and concentrated. The cleaved HPg was placed over a lysine-Sepharose 4B column (2.5 cm X 15 cm) (Brockway and Castellino, 1972) equilibrated with 50 mM Tris pH 8.0 until an absorbance at 280 nm of 0.05 was reached. The absorbed kringle fragments were then bulk eluted with 100% buffer of 50 mM Tris-HCl/300 mM  $\epsilon$ -amino caproic acid, pH 8.0. After dialysis of the kringle fractions against 20 mM Tris-HCl, pH 5.0, the material was applied to a BioRad Mono-S column equilibrated with the same buffer. The kringle portions, K4, K5, K1-3, K1-4 and K1-5 were eluted with a 0-20%, 20-50%, 50-70% and 70-100% step gradient of 20 mM phosphate to 20 mM phosphate /1 M KCl pH 5.0. K1-5 eluted at the last step gradient of the 70-100% step as determined by gel electrophoresis.. The molecular weights were determined by gel electrophoresis and Western Blot analysis with monoclonal antibodies specific for kringle 4, kringle 5, kringle 1-3 fragments and the protease domain.

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B. Method 2: Kringle fragment 1-5 (K1-5), was prepared from the limited digestion of Lys-HPg (Abbott Labs) with porcine pepsin (Sigma Chemical Co., St. Louis, MO). The Lys-HPg was added to a slurry of insoluble pepsin-agarose containing 100 mM  $\epsilon$ -amino caproic acid, pH 8.0 and stirred for 2 hours. The mixture was centrifuged at 3000 rpm to remove the pepsin agarose. The supernatant was dialysed against 50 mM PBS, pH 7.4 overnight. The sample was then placed over a lysine-Sepharose 4B column (Sigma)(2.5 cm X 15 cm) (Brockway and Castellino, 1972) equilibrated with 50 mM Tris pH 8.0 until an absorbance at 280 nm of 0.05 was reached. Kringle fragment K1-5 was eluted with 100% buffer of 50 mM Tris /300 mM  $\epsilon$ -amino caproic acid, pH 8.0. The sample was determined

by gel electrophoresis, and Western Blot analysis with monoclonal antibodies specific for kringle 4, kringle 5, kringle 1-3 fragments and the protease domain.